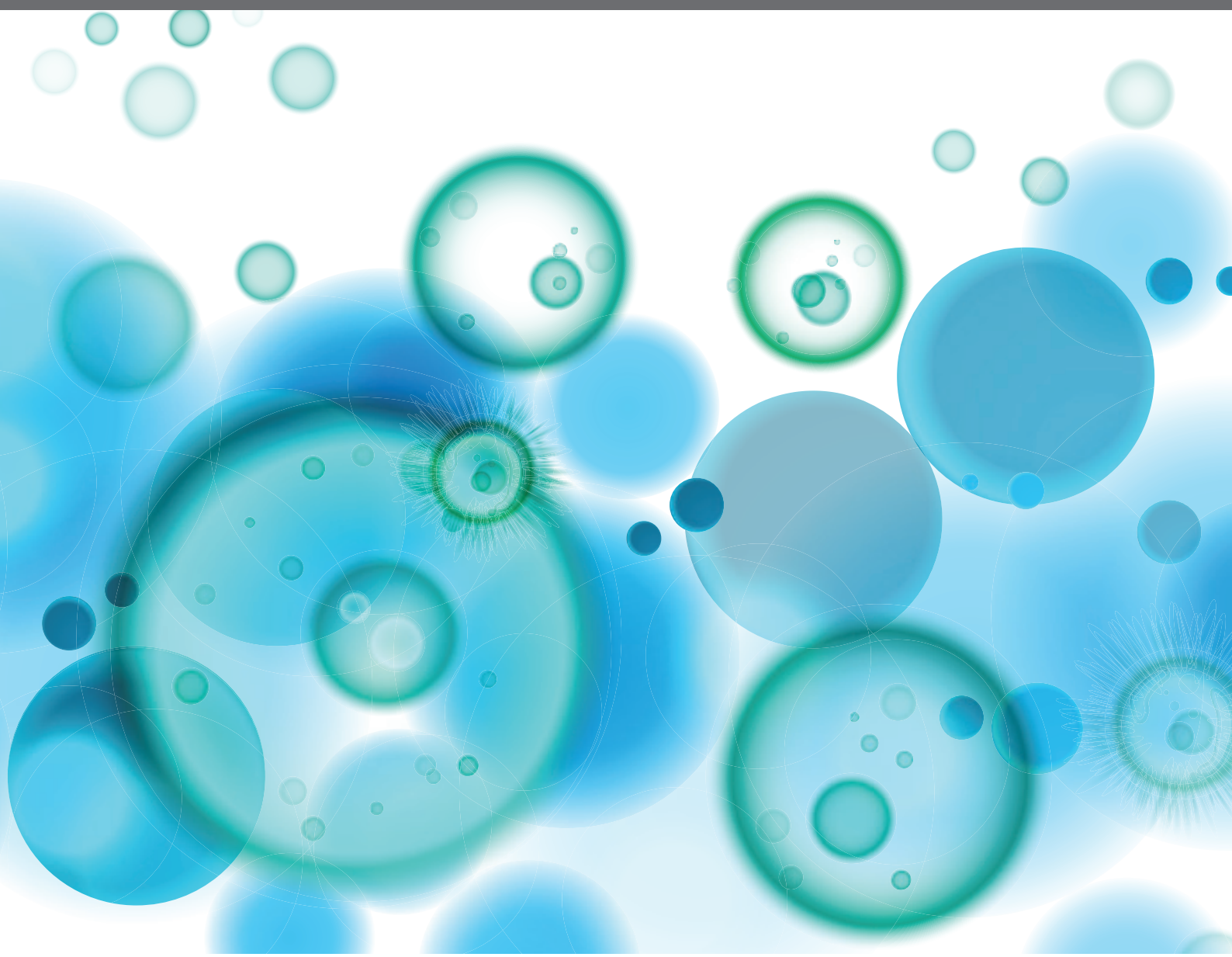
IMMUNE RESPONSES TO PERSISTENT OR RECURRENT ANTIGENS: IMPLICATIONS FOR IMMUNOLOGICAL MEMORY AND IMMUNOTHERAPY

EDITED BY: Alejandra Pera and Stefano Caserta
PUBLISHED IN: Frontiers in Immunology



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ISSN 1664-8714
ISBN 978-2-88966-718-5
DOI 10.3389/978-2-88966-718-5

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IMMUNE RESPONSES TO PERSISTENT OR RECURRENT ANTIGENS: IMPLICATIONS FOR IMMUNOLOGICAL MEMORY AND IMMUNOTHERAPY

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Citation: Pera, A., Caserta, S., eds. (2021). Immune Responses to Persistent or Recurrent Antigens: Implications for Immunological Memory and Immunotherapy. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88966-718-5

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frontiers in Immunology

EDITORIAL
published: 09 March 2021
doi: 10.3389/fimmu.2021.643989

Editorial: Immune Responses to Persistent or Recurrent Antigens: Implications for Immunological Memory and Immunotherapy

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Keywords: immunological memory, persistent antigens, immunosenescence, immunotherapy, cancer, sepsis, HIV, COVID-19

Editorial on the Research Topic

Immune Responses to Persistent or Recurrent Antigens: Implications for Immunological Memory and Immunotherapy

OPEN ACCESS

Edited and reviewed by:
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Specialty section:
This article was submitted to Immunological Memory, a section of the journal Frontiers in Immunology

Received: *19 December 2020*
Accepted: *01 February 2021*
Published: *09 March 2021*

Citation:
Caserta S and Pera A (2021) Editorial: Immune Responses to Persistent or Recurrent Antigens: Implications for Immunological Memory and Immunotherapy. Front. Immunol. 12:643989. doi: 10.3389/fimmu.2021.643989

Immunological memory [for a critical, wider analysis of this concept refer to (1) and (2)] is generally considered a hallmark of the adaptive immune response, which is essential for long-term protection against infection throughout life. From the perspective of adaptive immunity, clonally expanded antigen-specific lymphocytes (T and B cells) accumulate within the immunological memory repertoire to confer protection upon re-encounter with persistent and/or recurrent pathogens. Furthermore, memory cells often respond more rapidly and effectively following antigen (Ag) encounter than naïve precursors do. Recent increasing evidence suggests that immunological memory can also be a feature of innate immune cells (3). Innate immunological memory has been frequently described as a trained potentiation of anti-pathogen responses upon re-infection and is exquisitely coordinated by transient genetic and transcriptional changes (e.g., epigenetic reprogramming) that alter the functions of innate immune cells, such as macrophages, monocytes, dendritic cells, and NK cells (3). The articles in this collection mostly focus on adaptive memory/memory-like cell responses development during chronic/endemic Ag exposure with implications for aging, infection, cancer, and therapy (**Figure 1**).

Under physiological conditions, throughout life, immunological memory responses undergo alterations implicated in (or perhaps even driving) the aging process. In their original review, Aiello et al. describe how the immune system changes during aging, relevant for vaccination efficacy, and other therapies aimed at reversing/delaying immune cell aging. For example, the authors review the application of dietary antioxidants and anti-inflammatory compounds (carotenoids, polyphenols, and polyunsaturated fatty acids); calory restriction and its drug mimetics (e.g., metformin); and micronutrients (such as zinc and vitamins). Further, they analyze strategies to reverse immune cell aging in vulnerable individuals, discussing the potential use of IL-7, as a growth factor to sustain naïve T cells, and checkpoint inhibitors as enhancers of T-cell responses, during aging. They explore the effects of microbiota (and dysbiosis) in immunotherapy linking this to the use of probiotics/prebiotics to limit inflammation, potentially useful to control inflammageing. The authors then discuss intracellular signaling pathways (p38/MAPK, sestrins/AMPK, and mTOR pathways) that could become targets of drug inhibitors, useful to limit/reverse (immune cell) aging, and new adjuvant formulations to boost vaccination efficacy in the elderly. Some of the

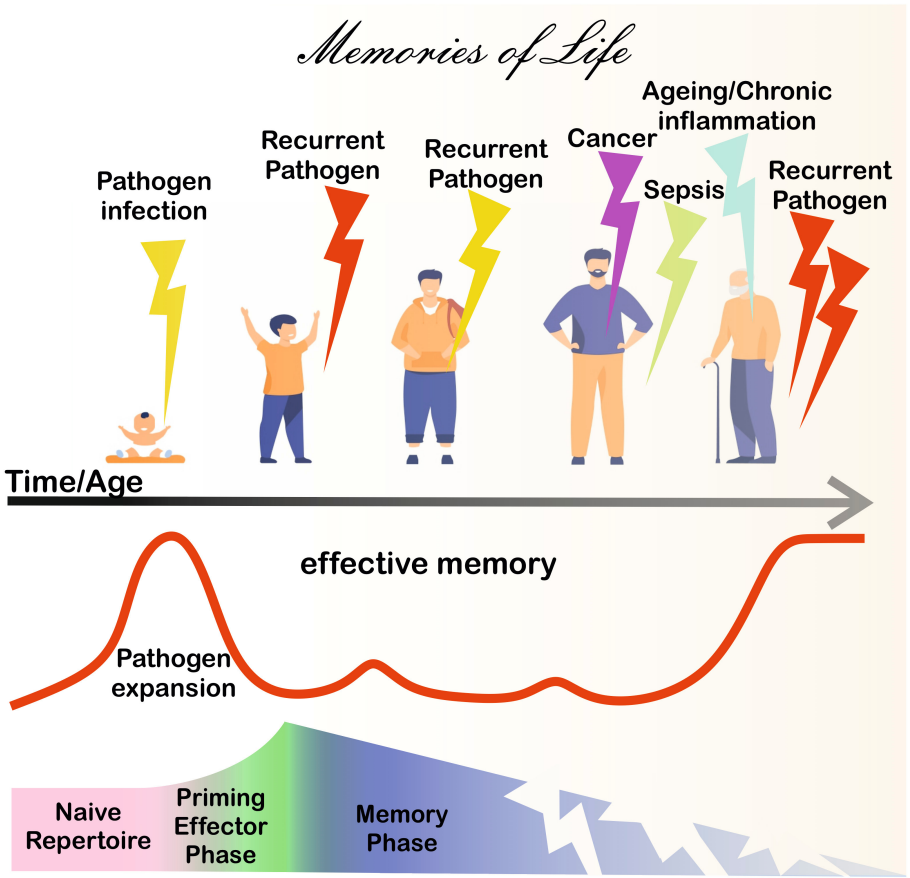

FIGURE 1 | Immunological memory during life. After birth, pathogen-derived antigens (Ags) prime adaptive immunity cells from naive precursors (pink shadowed area), eventually leading to the formation of immunological memory (blue shadowed area), after the effector phase (green shadowed area). Memory cells protect in the long-term, however the memory repertoire is continually exposed to chronic Ag sources. For instance, chronic/recurrent inflammation and/or infection due to the presence of persistent/endemic pathogens or microbial Ag (bold red line) is likely to occur throughout life. Cancer disease can further expose adaptive cells to chronic tumor Ags. Severe infections, such as sepsis and COVID-19 are associated with immunoparalysis/exhaustion and/or loss of T cells. Ultimately, chronic/persistent stimulants can lead to a deterioration of immunity, leaving individuals more vulnerable to disease.

changes associated with age are the result of adaption to stimuli, thus Aiello et al. stress the importance of targeted "rejuvenating" approaches. Indeed, memory responses to certain pathogens can deteriorate during aging, as seen for the age-associated decline of VZV-specific T cells. Contrarily, T-cell responses to other pathogens (most evidently, CMV) persist throughout life, reducing the diversity of the memory repertoire. Thus, both lifestyle and genetic factors influence immunosenescence and must be considered for treatment strategies.

The generation, persistence, and function of memory cells in humans during life may differ substantially from the responses seen in animal models where experimental variables are controlled in a reductionist approach, for example by deciding *a priori* the clonality of responder cells, the dose and modality of Ag exposure/administration, often without testing the impact of exposure to multiple infectious stimuli, as seen in real life conditions (4). Rather chronic/recurrent inflammation and/or infection due to the presence of persistent/endemic pathogens or microbial Ag are more likely to occur in humans, during life (1). Often (i) persistent or recurrent pathogens (e.g., virus, bacteria, and fungi); (ii) self-Ag derived from the body tissues; and (iii) cancer cells can lead to a deterioration of the immune response characterized by genetic/epigenetic alterations in immune cells driven by chronic or repeated exposure to Ags (**Figure 1**). In adaptive immunity, this constant stimulation by persistent Ags can lead to a disproportionate accumulation of Ag-experienced or memory-phenotype lymphocytes (memory inflation) (5). These phenomena can be associated with (i) a decreased diversity of Ag-receptor repertoires and (ii) alterations in signal transduction and cell differentiation processes, subsequently leading to dysfunctional responses, including exhaustion.

Relevant to Ag-receptor repertoires, in their original research article, Naumova et al. analyze the clonotype distribution within the circulating TCR-Vβ19^{+} CD8 T-cell pool (known to include

Abbreviations: Ag, antigen; ACT, adoptive T-cell therapy; CAR, chimeric antigen receptors.

influenza virus-specific T cells), during time. Under steady-state conditions (i.e., far from flu-like episodes), the blood T-cell repertoire comprises a dynamic component (potentially, tissue/depot resident cells entering the circulation) and many, low-frequency clone subpopulations which account for the larger fraction of the repertoire, stable over time. Such clonotype distribution may be reshaped by Ag-recall events in re-infected individuals, with repercussions on repertoire stability. Naumova et al. characterize the impact of Ag re-encounter on clonotype distribution in cultures derived from children, middle-age and older adults, at different time points. In terms of stability, the middle-age adults' repertoires are the most resilient, showing similar clonotype distributions between the recall and steady-state conditions. Hence, the generation of stable clonotypes in the repertoire relies on the maturation of the immune system over the years. In children, stable influenza-specific clonotypes are absent and recurrent (viral) Ag can drive loss of clonotypes that -the authors propose- would be replenished with new, best-fitting clonotypes. Toward adulthood, the T-cell repertoire would mature to reach an optimum of clonotype distribution and stability, capable to withstand Ag re-encounters, providing efficient protection against recurrent pathogens. However, such repertoire stability would erode in the face of repeated exposure to viral Ag, later in life. Especially in older individuals, the rate of clonotypes loss after recurrent infections would mark the deterioration of the repertoire and, hence, immunosenescence. This opens the interesting question as to how the skewness of the repertoire generated by real-life infections would impact on the responses, not only to the same, but also other pathogen-derived, cancer, and perhaps even self (cross-reactive?) Ags.

Covering cell differentiation processes in the context of chronic infections and persistent tumor Ags, Hope et al. present the challenges currently faced in the field to distinguish senescent and exhausted cells from memory counterparts. The authors discuss the surface markers (including inhibitory receptors: PD-1, TIM3, LAG-3, and others), the cytokines and the transcription factors (among many others: Blimp-1 vs. Bcl-6, Id2 vs. Id3, Eomes vs. T-bet, TOX, and Tcf-1) used to discriminate polyfunctional and/or long-term memory cells from the rest. They critically review memory-cell development/differentiation in patient infection studies and mouse models, from LCMV to SIV/HIV-1 and HCV, as well as *Trypanosoma cruzi*, *Toxoplasma gondii* and *Plasmodium* spp., and *Mycobacterium tuberculosis*, among others. They additionally draw important parallels with the case of chronic Ag responses during tumor disease, looking at melanoma, colorectal cancer, non-small cell lung carcinoma, and breast cancer. The changing horizons of T cell differentiation to chronic Ags can be decided by Ag load, time, and length of exposure, Ag removal and/or resolution of (including other) infection, cytokine milieu, concomitant inflammation, and anatomical cellular compartmentalization, with differences between CD8 and CD4 T cells. Further, CD4 T cells critically help memory CD8 T cell formation and B cell responses (especially in the case of T_{FH} responses), yet much more work is needed to characterize these during chronic infections and cancers. Therapeutically, in both cancer and infection, T-cell differentiation balances may be shifted with the application of antibodies directed to checkpoint receptors (anti-PD-1/PD-L1, anti-CTLA-4) to restore long-term responses, valid for both CD8 and CD4 T cells. In addition, vaccination with MHC-I/II-restricted Ag combined with specific adjuvants and/or concomitant depletion of regulatory T cells may prove beneficial to developing and maintaining responses to chronic tumor Ags.

Following on this thread, severe infectious conditions, such as sepsis, are also known to affect the metabolic profile and function of immune cells, somehow speeding up the exhaustion of memory-like cells. For example, patients affected by sepsis are more likely to have lifelong sequelae including the increased susceptibility to other subsequent infection (6), opening up the question of whether sepsis impacts on the memory immune repertoire with long-lasting impact. Relevantly, in their original research article, Niu et al. analyze inhibitory receptor expression in T cells during sepsis. They found that, in acute sepsis patients, an increased proportion of T cells express PD-1 *ex vivo* yet, later on (5 days from admission) these can further co-express LAG-3. This identifies a progression of T cell dysfunction/exhaustion during sepsis development. In recall responses to anti-CD3/CD28 and PMA/Ionomycin, sepsis-patient derived T cells that co-express both inhibitory receptors are less likely to secrete cytokines and proliferate, while showing increased trend to cell-death, relevant for sepsis immunoparalysis. Co-expression of PD-1 and LAG-3 on T cells is a relevant sepsis prognostic biomarker, as increased proportions of LAG-3^+ PD-1^+ T cells mark patients with more severe organ dysfunction, longer hospital stay, and diminished survival. Therapeutically, this study points at future avenues combining anti-PD-1 and anti-LAG-3 blockade, with specific timings to selectively prevent progression of exhaustion. Thus, future studies aimed at understanding the implications of T-cell differentiation and the wider impact that immunoparalysis can have on memory T cells in sepsis are critical to ameliorate therapy and manage patients, post-recovery.

Further on the theme of T-cell dysfunction in infection and cancer, the review by Vigano et al. discuss how T cell exhaustion is a common trait between HIV-1 infection and cancer. T-cell exhaustion is the consequence of Ag persistence, additionally instructed by immunoevasion mechanisms, particularly relevant in the tumor microenvironment. In both conditions, persistent activation induces TOX transcription factor which controls the transcriptional and epigenetic reprogramming of exhausted T cells. Another shared feature of T cell exhaustion in cancer and HIV-1 infection is the co-expression of several inhibitory immune checkpoint receptors (PD-1, CTLA-4, TIM-3, LAG-3, TIGIT, CD160, 2B4, and BTLA) by these cells. These changes translate into functional and survival defects that compromise T-cell effector functions and expansion capacity, while increasing susceptibility to apoptosis. Significantly, Vigano et al. cover differences affecting exhaustion in cancer and HIV-1 infection. For example, in HIV-1 infection, exhausted CD8 T cells express T-bet and Eomes which distinguish these cells from their progenitors, while in cancer the key transcription

factors associated with exhaustion are Tcf-1 and STAT3. Similar to chronic viral infection, in cancer exhausted T cells have impaired functions. However, tumor infiltrating T cells are heterogeneous and can retain some degree of functionality that contribute to tumor control. This may explain the highly variable outcomes of immune checkpoint inhibitors therapy. Finally, the authors highlight the importance of discerning exhausted T cells from memory and activated cell phenotypes in order to design targeted immunotherapies. In this respect, inhibitory receptor expression is higher on exhausted rather than activated effector T cells, which express these receptors transiently. In addition, the transcriptional profiles of memory and exhausted T cells differ (*Rtp4, Foxp1, Ikzf2, Zeb2, Lass6, Tox,* and *Eomes*) and, in specific loci (e.g., *Pdcd1*), chronic Ag drives exhaustion-associated epigenetic imprinting that cannot reverse even after Ag decrease/removal. Understanding the processes involved in T-cell exhaustion during persistent stimulation by cancer or viral Ags is essential for the development of new immunotherapies.

With this in mind, the topic then develops into the therapeutic implications of immune memory for adoptive immunotherapy of cancer. In their review, Mondino and Manzo address the impact that pre-existing memories within the T-cell repertoire can have on the efficacy of cancer immunotherapy, in particular focusing on adoptive T-cell therapy (ACT), including chimeric Ag receptors (CAR) T cell therapy. During life, successive exposure to various Ags shapes the composition of memory (or Ag-experienced) immune repertoires in individuals. Bystander, cross-reactive, unrelated and/or suppressive memories instructed throughout the personal history of pathogen exposure will impact on future endogenous memory responses to cancer, as well as the efficacy of ACT. This would point at potential competition for environmental cues between endogenous pre-existing memory T cell clones and the transplanted cells. Factors to be considered in this respect span from clonal abundance and TCR affinity/avidity to availability of cytokines (particularly, common-γ chain cytokines) and nutrients (glucose, amino acids, and fatty acids). The authors discuss that pre-existing memory cells generated in response to previously encountered pathogens or cancer Ags in the initial stages of the disease could synergize with adoptively transferred T cells, in specific conditions. Yet, certain pre-existing memory cells could well-undermine the engraftment and dampen the efficacy of ACT cells, especially if they were to share characteristics of regulatory T cells. Authors propose that "good memories" will be cells with effector capabilities able to synergize with tumor-specific T cells provided by ACT. In this respect, proper activation of certain viral-specific memory T cells, could synergize with ACT. In contrast, "bad memories" would impair the development of new memories. Thus, repetitive encounters with the Ag could generate both good and bad memories, with opposite effects on the efficacy of ACT therapy. A thorough characterization of the host immunocompetence might help improving the efficacy of T-cell products, therefore increasing the probabilities of a successful therapeutic outcome.

The recent example of SARS-Cov-2 infection that has become endemic in the world is posing several interesting questions around memory cell persistence and function, in affected individuals. Although multiple vaccines may soon become widely available to hopefully protect against COVID-19, infection with SARS-Cov-2 is emerging as a new variable, drastically impacting on immune cells (7), potentially with long-term consequences for immunity. In their article, Diao et al. clearly show that immune cells, and in particular T cells are vastly reduced in total numbers in progressive COVID-19 disease stages. Such T-cell reduction is reminiscent of that happening in HIV^+ individuals, but importantly further extends to CD8 T cells. This general loss of T cells would also affect individuals with mild infection symptoms and/or not requiring hospitalization. Interestingly, in COVID-19 patients, T-cell numbers inversely correlate with levels of inflammatory cytokines, IFN-γ, IL-6, and TNF-α, often described during cytokine storm reactions seen in sepsis and other systemic inflammatory diseases. The authors also show that, in the most severe forms of COVID-19, T cells would acquire an exhaustion phenotype. It remains unclear whether recruitment of T cells in other anatomical compartments (e.g., the lungs?) may explain loss of T cells during COVID-19. Nonetheless, drastic changes such as those described by Diao et al. would likely have an impact on the memory T cell compartment, potentially affecting, or even undermining, efficient responses to future pathogen encounters. Thus, it remains to be determined whether the phenomenon described by Diao et al. would help erase, change, and/or unbalance the historical record of immune memories within the repertoire of post-COVID-19 patients. Speculatively, it could be anticipated that such changes may play a role in long-COVID (8), and perhaps in future responses to cancer and recurrent pathogen Ags that post-COVID-19 patients will experience, later in life after recovery. Conversely, the weakening of the immune system so well-described in the elderly (Aiello et al.), and in patients suffering from cancer/HIV (Hope et al. and Vigano et al.) or sepsis (see Niu et al.), may predispose these individuals to severe COVID-19 disease, upon SARS-Cov-2 infection. It is still unclear whether individuals who experience non-severe or no symptoms after SARS-Cov-2 exposure might have cross-reactive memory T cells capable of mediating protection against this coronavirus (7). Recent evidence suggests the existence of cross-reactive Ags derived from other (more or less endemic) coronaviruses which may provide some degree of protection upon infection with SARS-Cov-2 (9). Again, fitting with this article collection, this would point at how recurrent exposure to endemic pathogens potentially helps create, shape, and even destroy our memories (**Figure 1**) with critical implications for health and disease, and treatment strategies.

AUTHOR CONTRIBUTIONS

SC and AP wrote the first manuscript draft and critically contributed to the final version of the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

SC was supported by the University of Hull. AP was supported by Miguel Servet CP19/00008 contract from Instituto de Salud Carlos III co-funded by ERDF/ESF, A way to make Europe/Investing in your future. The funders had no role in the decision to publish or preparation of the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 17 July 2019
doi: 10.3389/fimmu.2019.01595

Striking a Balance—Cellular and Molecular Drivers of Memory T Cell Development and Responses to Chronic Stimulation

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Effective adaptive immune responses are characterized by stages of development and maturation of T and B cell populations that respond to disturbances in the host homeostasis in cases of both infections and cancer. For the T cell compartment, this begins with recognition of specific peptides by naïve, antigen-inexperienced T cells that results in their activation, proliferation, and differentiation, which generates an effector population that clears the antigen. Loss of stimulation eventually returns the host to a homeostatic state, with a heterogeneous memory T cell population that persists in the absence of antigen and is primed for rapid responses to a repeat antigen exposure. However, in chronic infections and cancers, continued antigen persistence impedes a successful adaptive immune response and the formation of a stereotypical memory population of T cells is compromised. With repeated antigen stimulation, responding T cells proceed down an altered path of differentiation that allows for antigen persistence, but much less is known regarding the heterogeneity of these cells and the extent to which they can become "memory-like," with a capacity for self-renewal and recall responses that are characteristic of *bona fide* memory cells. This review focuses on the differentiation of $CD4^+$ and $CD8^+$ T cells in the context of chronic antigen stimulation, highlighting the central observations in both human and mouse studies regarding the differentiation of memory or "memory-like" T cells. The importance of both the cellular and molecular drivers of memory T cell development are emphasized to better understand the consequences of persisting antigen on T cell fates. Integrating what is known and is common across model systems and patients can instruct future studies aimed at further understanding T cell differentiation and development, with the goal of developing novel methods to direct T cells toward the generation of effective memory populations.

Keywords: T cell memory, cancer, chronic infection, CD4 T cells, CD8 T cells

OPEN ACCESS

Edited by:
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Reviewed by:
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Specialty section:
This article was submitted to Immunological Memory, a section of the journal Frontiers in Immunology

Received: *01 March 2019*
Accepted: *26 June 2019*
Published: *17 July 2019*

Citation:
Hope JL, Stairiker CJ, Bae E-A, Otero DC and Bradley LM (2019) Striking a Balance—Cellular and Molecular Drivers of Memory T Cell Development and Responses to Chronic Stimulation. Front. Immunol. 10:1595. doi: 10.3389/fimmu.2019.01595

INTRODUCTION

T cells are essential for the adaptive immune system's responses to pathogens and tumors. They are vital for the clearance of host cells that become infected with viruses and intracellular bacteria as well as the elimination of tumor cells (1, 2). T cell memory is typically defined as a residual compartment of protective antigen-specific T cell that persists long after contraction of the effector

pool and survives in the absence of antigen (3). It is an important distinction that antigenic withdrawal does not occur during chronic infections and cancer (**Figure 1**) despite prolonged survival of responding T cells. Therefore, further insights are required into the differentiation of T cells in these contexts and in settings where clearance of a once chronic antigen ultimately occurs. These include (1) identifying characteristic phenotypic markers and transcriptional profiles, (2) ascertaining the capacity for self-renewal, and (3) determining the ability for rapid re-activation and generation of polyfunctional responses (4, 5). The focus of this review is to highlight the known differences in memory $CD4^+$ and $CD8^+$ T cell development in the context of chronic pathogen infections or cancer progression as compared to acute infections in both mice and humans, with an emphasis on the cellular and molecular drivers of T cell memory development under these conditions.

During the primary T cell response to infection or tumors, the antigen-specific T cell pool becomes highly heterogeneous, forming different subpopulations of $CD4^+$ and $CD8^+$ T cells defined by surface marker expression, transcription factors, cytokine production, and cytotoxic or memory-forming potential (**Figure 1**). Ultimately, with antigen clearance, the large majority of antigen-specific T cells die and a smaller pool of memory T cells that retain the capacity to respond to re-challenge can persist, often indefinitely (6, 7). However, memory T cells are also highly diverse, with substantial differences described for a variety of infections, implying the importance of contextual cues such as the duration of antigen exposure as well as the tissue localization and distribution of infection. Much less is known about the differentiation of memory $CD4^+$ T cells compared to $CD8^+$ T cells, in part because of the ability of naïve $CD4^+$ T cells to adopt different effector cell fates that are uniquely regulated and are elicited by different infections. However, studies of circulating memory $CD4^+$ (and $CD8^+$) T cells in humans were the first to define effector memory T cells (T_{EM}: $CD45RA^+CD127^+CD62L^-$/$CCR7^-$), and central memory cells (T_{CM}: $CD45RA^+CD127^+CD62L^+$/$CCR7^+$) that primarily differ with respect to the circulation through secondary lymphoid organs and the capacity for self-renewal ($T_{CM} > T_{EM}$) (8). In contrast to the extensive literature on the development of T cell memory to infections, much less is known about the characteristics of memory T cells that develop in responses to tumors.

There is considerable diversity in the fates of a naïve T cell and the mechanisms regulating the formation and promotion of heterogeneity in effector and memory T cell pools are of great interest, particularly in the context of vaccine development. Several models of memory T cell differentiation have been proposed and previously discussed elsewhere, but there is currently robust evidence for the "one cell, multiple fates" model (9, 10). In mice, fate-mapping and memory differentiation of $CD8^+$ T cells that were assessed by Dirk et al. by performing adoptive transfer of single naïve OT-I TCR transgenic $CD8^+$ T cells into recipient mice, followed by infection with OVA-expressing bacterium *Listeria monocytogenes* (Lm-OVA) (11, 12). These studies conclusively demonstrated that a naïve T cell could subsequently differentiate into both effector and memory T cells. Another study from Schumacher et al also assessed memory $CD8^+$ T cell differentiation by fate-mapping analysis of adoptively transferred T cells, but used DNA-barcoded, transduced thymocytes from OT-I mice that were injected intra-thymically into young recipients, followed by infection with Lm-OVA (10). This study showed that a single antigen-specific naive $CD8^+$ T cell gave rise to daughter cells with multiple phenotypes, including T_{CM} and T_{EM} subsets. Furthermore, the progeny of a single naïve $CD8^+$ T cell could efficiently seed the secondary lymphoid organs (bone marrow, blood, spleen, and lymph nodes) and were not restricted to a particular anatomical location. Importantly, barcoded memory $CD8^+$ T cells that were transferred into tertiary hosts maintained barcode diversity upon re-challenge, indicating the potential for all clones to respond. The fundamental question of whether effector T cells can give rise to memory cells was also demonstrated by fate mapping studies in which effector $CD8^+$ T cells, identified by acquisition of the cytotoxic protein granzyme B, were shown to form memory (13). A more recent study demonstrated the ability of effector $CD8^+$ T cells to "de-differentiate" into memory T cells by epigenetic remodeling associated with alterations in the DNA methylation programs of the cells (14). Together, these groups and others have demonstrated that indeed one naïve $CD8^+$ T cell has the potential to give rise to daughter cells with differing phenotypes and fates, and that effector differentiation does not preclude memory development. However, to our knowledge such comprehensive studies addressing $CD4^+$ T cell memory development have yet to be published and is a significant gap in understanding overall T cell memory differentiation.

Signals determining memory T cell generation remain incompletely understood. It is evident that antigen availability and timing of entry into a response are important determinants for memory formation. In general, weaker TCR signals are

Abbreviations: T_N, Naive T cell; T_{EFF}, Effector T cell; T_{EX}, Exhausted T cell; T_{CM}, Central memory T cell; T_{EM}, Effector memory T cell; T_{RM}, Resident memory T cell; T_{SC}, Stem-cell like T cell; T_{EMRA}, Terminally differentiated effector memory T cell; OVA, Ovalbumin; Lm-OVA, *Listeria monocytogenes*-OVA; OT-I, Transgenic mouse $CD8^+$ T cells that recognize OVA (SIINFEKL); TCR, T cell receptor; IFN, Intereferon; IL, Interleukin; T-bet, T-box transcription factor TBX21; Eomes, Eomesodermin; HSV, Herpes simplex virus; RSV, Respiratory syncytial virus; Blimp-1, B lymphocyte-induced maturation protein-1; Bcl-6, B-cell lymphoma 6; Bcl-2, B-cell lymphoma 2; Foxo, Class O of forkhead box transcription factors; FRC, Fibroblastic reticular cells; PD-L1, Programmed death-ligand 1; PD-1, Programmed cell death protein 1; CTLA-4, Cytotoxic T-lymphocyte-associated protein 4; HIV-1, Human immunodeficiency virus 1; HCV, Hepatitis C virus; T_H1, Type 1 helper cell; IFN-γ, Interferon gamma; TNF-α, Tumor necrosis factor alpha; LCMV, Lymphocytic choriomeningitis virus; EBV, Epstein-Barr virus; CMV, Cytomegalovirus; HLA-I, Human leukocyte antigen, class I; CD, Cluster of differentiation; TB, Tuberculosis; LTBI, Latent tuberculosis infection; Hobit, Homolog of Blimp-1 in T cells; Tcf-1, T-cell factor 1; KLRG1, Killer cell lectin like receptor G1; CX3CR1, C-X3-C motif chemokine receptor 1; LAG3, Lymphocyte activating 3; ICOS, Inducible T-cell costimulator; T_{FH}, T follicular helper cells; T_{17}, T-helper 17 cells; SIV, Simian immunodeficiency virus; TIM3, T-cell immunoglobulin and mucin-domain containing-3; TIGIT, T cell immunoreceptor with Ig and ITIM domains; NSCLC, Non-small-cell lung carcinoma; scRNA-seq, Single cell RNA-sequencing; HCC, Hepatocellular carcinoma; TGF-β, Transforming growth factor beta 1; DC, Dendritic cell; MHC-I, Major histocompatibility complex class I; MHC-II, Major histocompatibility complex class II; GM-CSF, Granulocyte-macrophages colony-stimulating factor; FAO, Fatty acid oxidation; OXPHOS, Oxidative phosphorylation.

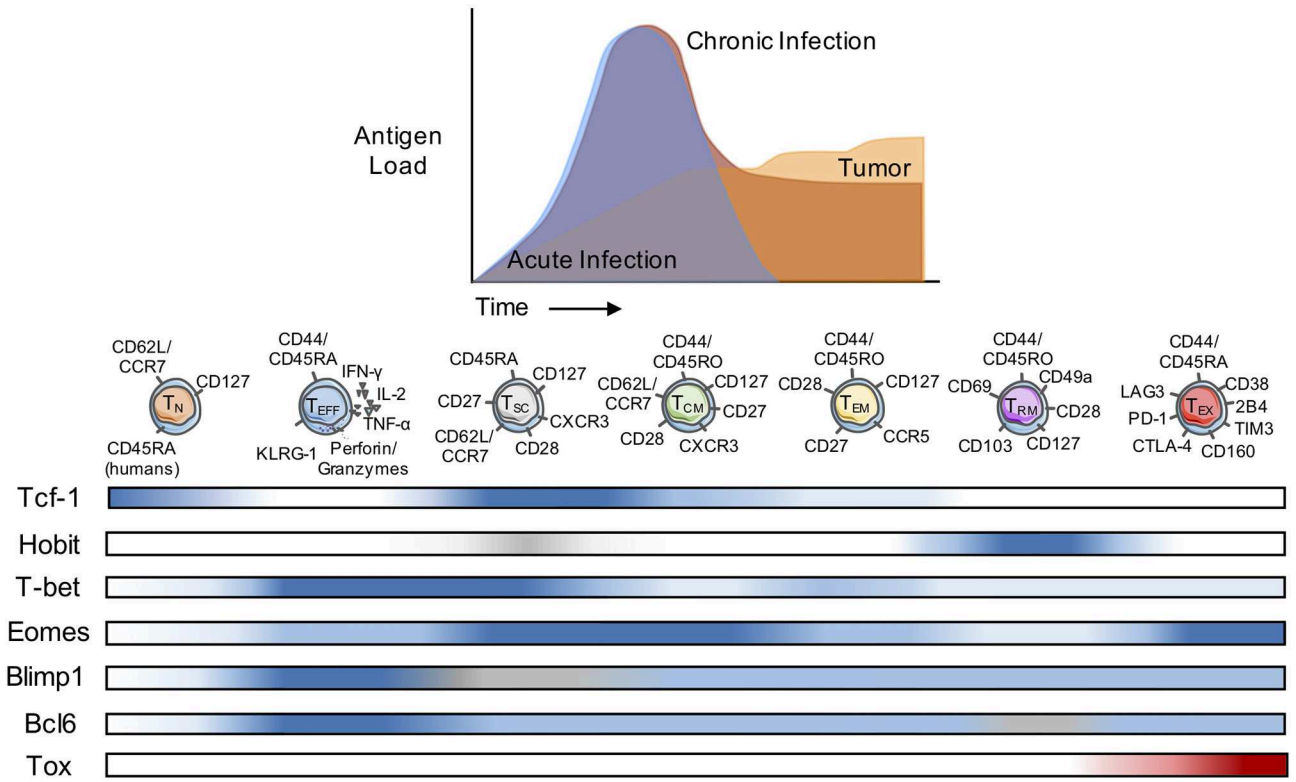

FIGURE 1 | Cellular and Molecular Dynamics of $CD8^+$ T Cell Responses. Naïve $CD8^+$ T cells (T_N) differentiate into diverse subsets with unique patterns of transcription factor (TF) expression and corresponding cell surface markers. The diversity of subset differentiation is highly influenced by the antigen or pathogen load in mice and patients. During acute infection, T_N cells give rise to polyfunctional, highly-proliferative effector (T_{EFF}) $CD8^+$ T cells that clear intracellular pathogens. Following contraction of T_{EFF} after antigen clearance, memory (T_{CM}, T_{EM}, and T_{RM}) $CD8^+$ T cells persist and rapidly respond upon re-infection. During chronic infection or in response to tumors, T_{EFF} also arise from Naïve, but often fail to effectively clear the infection or tumor and in response to persistent antigen can develop into T_{EX} with reduced function. In mice and patients, T_{SC} have been identified as a population of $CD8^+$ T cells that respond to checkpoint blockade therapy. The development of classically-defined T_{CM}, T_{EM}, and T_{RM} during chronic infection or cancer remains contested and the differentiation of "memory-like" T cell populations is discussed within this review. Blue, level of expression of TF; white, no expression; gray, expression unknown; red, characteristic expression in T_{EX}.

thought to favor memory T cell development, which can be influenced by TCR affinity, tissue localization with respect to antigen distribution, or by progressive antigen clearance (15). For $CD8^+$ T cells, there is evidence that unique TCR signaling and organization of the TCR signaling complex that engages NF-kB signaling dictates memory development (16). These findings along with observations that $CD8^+$ memory T cell precursors can be distinguished early in responses to acute infections in some models (e.g., LCMV Armstrong and *L. monocytogenes*) support the concept that early events are critical for memory development. Other external signals such as from cytokines during the effector phase also contribute to memory T cell differentiation. For example, type I interferon (IFN) or IL-2 signaling is key to the sustained survival of $CD8^+$, and $CD4^+$ T cells, respectively, during memory formation during the primary response (17, 18). Signaling through CD28 is required for the re-activation of memory $CD8^+$ T cells and optimal recall responses of memory $CD4^+$ T cells (7, 19). Co-stimulation of T cells such as through CD28 enhances their survival and effector function by increasing expression of the anti-apoptosis regulator $BCL\text{-}_{XL}$, as well as by inducing T cell expansion, by the production of IL-2 (20, 21). Cytokines that include type I IFNs and IL-12 induce changes in the transcription factors T-box expressed in T cells (T-bet) and Eomesodermin (Eomes) (22, 23), which play important roles in regulating effector and memory T cell differentiation (24, 25) as summarized below. $CD4^+$ and $CD8^+$ T cells also influence each other during memory T cell development. Although $CD4^+$ T cells are dispensable for the generation of primary effector $CD8^+$ T cell responses to some infections, CD27 on $CD8^+$ T cells interacting with CD70 on APCs primed by $CD4^+$ T cell "help" via CD40(APC)/CD40L($CD4^+$ T cell) activation is required for the generation of functional memory $CD8^+$ T cells marked by reduced proliferative capacity during recall responses (26).

Following a primary adaptive immune response, distinct subsets of memory T cells are found within the lymphoid organs that include not only T_{CM} or T_{EM}, but also more recently defined memory cells that become resident in the initial site of the primary infection or tumor (T_{RM}). All three subsets play

roles in protective memory responses, although T_{RM} are likely to provide a first line of defense against a tissue localized re-infection. The T_{RM} compartment was first characterized in the skin where these cells control reinfection with herpes simplex virus (HSV), and have since been identified as key mediators of immunity in the lung, such as in response to RSV and influenza; and in the gut after infection with Lm or LCMV (27–29). Although T_{RM} have been identified by phenotype in tumors, their functions are not yet established (30). The T_{RM} pool contains two subsets distinguished by CD103 expression (also known as integrin alpha E), a receptor for E-cadherin (31). Current studies are focusing on possible functional differences between the $CD103^+$ and $CD103^-$ subsets of T_{RM}. Another recently-defined memory T cell subset is considered to have stem cell-like properties with respect to self-renewal, and has been designated stem cell memory T cells (T_{SCM}). Unlike other memory $CD8^+$ T cell subsets, these cells maintain a naïve-like phenotype yet have a high proliferative capacity (32). Surface marker expression remains one of the primary methods for the classification of these different memory T cell subpopulations in both humans and mice (**Table 1**), and several distinctions apply more narrowly to memory cells generated in responses to specific infections. The presence of many of phenotypic and functional distinctions of memory cells has been much less well-studied in anti-tumor responses. It is now evident that a spectrum of surface phenotypes can arise primarily because of contextual cues and that these may be fluid and insufficient to fully define memory T cell subsets. However, in combination with analyses of transcription factor expression, greater insights into distinct features of memory T cell fates emerge.

Transcription factors are well-recognized as key regulators of T cell fate determination. In both $CD4^+$ and $CD8^+$ T cells, opposing levels of transcription factor pairs can strongly correlate with the T cell memory subsets. Examples of these gradients of transcription factors are T-bet vs. Eomes, B lymphocyte-induced maturation protein-1 (Blimp-1) vs. B-cell lymphoma 6 (Bcl-6), and Inhibitor of DNA binding 2 (Id2) vs. Inhibitor of DNA binding 3 (Id3) (65, 74, 75). At the memory stage, Eomes is more highly expressed than T-bet. Similarly, while Blimp-1 is highly expressed in effector cells and reciprocally Bcl-6 expression increases in memory cells; a similar relationship has been observed between Id2 and Id3. There is also a supportive role for transcription factor expression during early differentiation in memory formation and maintenance, specifically the Forkhead box proteins O-class proteins (Foxo) Foxo1 and Foxo3 (59, 76). Understanding how these transcription factors interact with each other remains an active area of research; for example, Foxo1 is known to regulate other transcription factors such as by increasing Tcf-1, Eomes, and Bcl-2 expression, while repressing the levels of T-bet. Within memory T cell subsets, there are also unique expression patterns of transcription factors, several of which are outlined in **Table 1** and highlighted in **Figure 1**. These transcription factors are altered in exhausted T cells (T_{EX}), and interestingly, expression patterns of some transcription factors associated with memory T cell formation are shared with T_{EX} that persist during chronic infection or cancer suggesting that re-stimulation of more "memory-like" T cells could contribute to achieving a balance between terminal T cell differentiation and pathogen or tumor control and the extent of T cell exhaustion, which has been extensively reviewed elsewhere (77).

Changes in the stimulatory conditions encountered by effector T cells could also impact the development of memory T cells, as reduced inflammation caused by increasing antigen control could limit the extent of the effector T cell response, particularly in the infection or tumor the draining lymphoid tissues. Thus, conditions that are highly influenced by: (1) the cellular microenvironment, and (2) changes in the molecular regulation of the responding cells are likely to be key in determining whether T cells become terminally differentiated or "memory-like" and thus lead to a spectrum of functionally heterogeneous T cells. An important consideration when contrasting T cell differentiation and development in responses to chronic infection or tumors is the influence of a highly systemic inflammatory response observed in some chronic infections and model systems compared to the more localized microenvironment typically associated with the early stages of cancer. However, there is evidence in both humans and mice of memory or "memory-like" $CD4^+$ and $CD8^+$ T cell formation under conditions of repeated stimulation. The following sections will outline the effects that persistent pathogenic infections or tumors have on $CD4^+$ and $CD8^+$ memory or "memory-like" T cell development and responses, and the interplay between the two.

T CELL RESPONSES TO CHRONIC INFECTION

$CD8^+$ Memory T Cell Development in Chronic Infection

The defining cellular environment of memory $CD8^+$ T cells is a compilation of interactions with other cellular compartments and the localization of the cell (such as in circulation, lymphoid tissues, or non-lymphoid tissues). In both acute infections and upon re-challenge, secondary lymphoid organs such as the peripheral lymph nodes are important sites of naïve or memory $CD8^+$ T cell activation during systemic viral infections such as LCMV. Here, dendritic cells (DC) that have captured and present LCMV antigens activate $CD8^+$ T cells. Surrounding the interacting T cell-DC conjugates are fibroblastic reticular cells (FRC), which can either promote and accelerate T cell activation, or conversely can inhibit subsequent expansion within the lymph nodes via the production of nitric oxide (78). FRC also constitutively express PD-L1, the ligand for the T cell inhibitory receptor PD-1 (programmed cell death protein 1, CD279) (79). During chronic LCMV infection, PD-L1 expression on FRCs is elevated and the network that supports T cell migration is disrupted, leading to altered localization (80). These changes in the lymphoid tissue architecture are thought to promote T cell exhaustion and impede memory formation. Persistent viral infections, such as LCMV in mice and Human Immunodeficiency Virus-1 (HIV-1) and Hepatitis C Virus (HCV) in humans, can also result in the inhibition and loss of type 1 T helper (T_H1) $CD4^+$ T cell responses that play a major role in supporting

TABLE 1 | Expression profiles of $CD8^+$ T cell subsets.

	T_N	T_{Eff}	T_{EMRA} (Hu)	T_{CM}	T_{EM}	T_{EX}	T_{RM}	T_{SC}
SURFACE MARKERS								
CD62L	+ (33)	− (34, 35)	− (36)	+ (34, 35)	− (34, 35)	− (34)	− (31)	+ (37)
CCR7	+ (38)	− (34)	− (36)	+ (34, 35, 38)	− (34, 38)	− (34)	± (38)	+ (37)
CD44	− (33)	+ (33)	N/A	+ (35)	+ (35)	+	+	(low) (37)
CD45RA (Hu)	+ (39)	+ (39)	+ (39)	− (39)	− (39)	−	−	− (37)
CD45RO (Hu)	− (39)	− (39)	− (39)	+ (35, 39)	+ (35, 39)	+	+	+ (37)
CD122 (IL-2R β-chain)	− (33)	+ (33)	N/A	+ (33, 35)	+ (33)	− (33)	− (33)	+ (37)
CD127 (IL-15R)	+ (40)	− (40)	+ (40)	+ (35, 40)	+ (35, 40)	+	± (41)	+ (37)
CD27	+ (40, 42)	− (40, 42, 43)	± (39, 40)	+ (40)	+ (40)	N/A	± (41)	+ (37)
CD28	+ (40, 42)	− (42)	− (39, 40)	+ (40)	+ (40)	N/A	± (41)	+ (37)
KLRG1	− (40, 43)	+ (35, 40, 43)	+ (40)	− (37, 40)	+ (37, 40)	N/A	N/A	− (37)
CXCR3	− (44)	± (44, 45)	N/A	+ (44, 45)	−(44)	N/A	(low) (46)	+ (37)
PD-1 (CD279)	− (47)	+ (47)	± (40)	± (40, 47)	± (40, 47)	+ (47, 48)	± (41)	+ (49)
CTLA-4 (CD152)	− (50)	+ (50)	N/A	(low) (50)	(low) (50)	+ (35, 48)	N/A	N/A
LAG-3 (CD233)	− (50)	N/A	N/A	+	+	+ (35, 48)	N/A	N/A
TIM-3 (CD366)	− (50)	(low) (50)	N/A	N/A	N/A	+ (35, 48)	N/A	N/A
2B4 (CD244)	− (50)	− (51)	N/A	N/A	N/A	+ (48)	N/A	N/A
CD160	− (50)	− (51)	N/A	N/A	N/A	+ (48)	N/A	N/A
CD69	− (52)	+ (35)	N/A	− (38)	− (38)	+ (48)	+ (31, 38)	+ (49)
CD103	N/A	N/A	N/A	− (38)	− (38)	N/A	± (38)	N/A
Sca1	N/A	N/A	N/A	N/A	N/A	N/A	N/A	+ (37)
CXCR5	− (50)	N/A	N/A	N/A	N/A	− (49)	N/A	+ (49)
CYTOKINES/CYTOTOXIC GRANULES								
IL-2	− (53)	+ (48)	+ (36)	+ (51)	+ (51)	− (48)	+ (41)	+ (54)
IFNγ	− (50)	+ (48)	+ (36)	+ (51)	+ (51)	− (48)	+ (41)	+ (54)
TNFα	− (53)	+ (48)	N/A	+ (51)	+ (51)	− (48)	+ (41)	+ (54)
Perforin	− (53)	+ (48)	+ (55)	(low) (51)	− (51)	− (48)	N/A	(low) (54)
Granzyme B	− (50)	+ (48)	+ (36)	(low) (51)	+ (51)	− (48)	+ (41)	(low) (54)
TRANSCRIPTION FACTORS								
Tcf1	(high) (56)	− (57)	± (56)	(med) (35, 56)	(low) (56)	− (56)	− (58)	(high) (49)
Foxo1	(high) (59)	+ (59)	+ (59)	+ (59)	+ (59)	+ (59)	N/A	N/A
Runx3	+ (60)	+ (35, 60)	N/A	+ (60)	+ (60)	+ (61)	+ (62)	N/A
ID2	− (50)	+ (35, 50)	N/A	N/A	+ (63)	+ (50)	N/A	N/A
ID3	+ (50)	+ (50)	N/A	+ (35, 63)	N/A	+ (50)	N/A	N/A
Tbet	− (50)	(high) (35)	+ (64)	(low) (35)	(med) (35)	(low) (35)	(low) (63)	+ (37)
Eomes	− (50)	(med/hi) (35)	N/A	(high) (35)	(med) (35)	(high) (35)	(low) (63)	+ (37)
Blimp1	− (50)	(high) (35)	N/A	+ (50)	+ (35, 50)	+ (35, 50)	+ (63)	N/A
Bcl6	− (65)	+ (65)	N/A	+ (35)	+ (35)	+ (57)	N/A	+ (57)
IRF4	− (66)	+ (66)	N/A	(low) (50)	(low) (50)	(high) (50)	(low)	(low)
BATF	− (50)	(low) (50)	N/A	− (50)	− (50)	+ (35, 50)	N/A	N/A
Hobit	− (67)	− (67)	+ (68)	− (67)	− (67)	− (67)	+ (63, 67)	N/A
Tox	− (50)	− (50)	N/A	− (50)	− (50)	+ (50, 69–73)	− (69)	N/A

Denotes expression observed primarily in mice.

N/A Denotes expression either unknown or not discussed within this review.

memory $CD8^+$ T cell development (81–83). During LCMV chronic infection, there is a progressive decline in virus-specific $CD8^+$ T cells; however, reconstituting the T_H1 $CD4^+$ T cell compartment is sufficient to maintain the LCMV-specific $CD8^+$ T cell population, providing greater evidence for a supportive role for $CD4^+$ T cells in maintaining long-lasting $CD8^+$ T cells that continue to undergo progressive differentiation (83).

Localization of virus-specific $CD8^+$ T cells in other non-lymphoid sites such as the kidney, liver, and lungs during chronic LCMV infection has been previously described, with evidence that they too exhibit signs of functional

exhaustion with decreased IFN-γ and TNF-α production upon *ex vivo* stimulation compared to conventional LCMV-specific memory $CD8^+$ T cells (84). Similarly, in a chronic parasitic infection model of *Trypanosoma cruzi* in mice, muscle-resident $CD8^+$ T cells have decreased effector function (85). The majority of long-lived $CD8^+$ T cells in the lung, liver, and kidneys after chronic LCMV infection fail to express CD103; however, LCMV-specific intraepithelial $CD8^+$ T cells found within the small intestine and lamina propria express both CD103 and CD69 (86), which establishes tissue localization via the G-protein-coupled receptor sphingosine-1-phosphate receptor (S1PR1) (87). However, whether these T_{EX} in non-lymphoid tissues share features with T_{RM} or provide a major role in maintaining chronic infection has not been studied.

While our understanding of $CD8^+$ T cell differentiation and "memory-like" development during chronic infections has largely been derived from mouse model systems, several studies have focused on dissecting human virus-specific $CD8^+$ T cell differentiation under persistent viral infections including HIV-1, HCV, Epstein Barr virus (EBV), and cytomegalovirus (CMV) through the use of Human Leukocyte Antigen class I (HLA-I) tetramers complexed with peptides of virus-derived $CD8^+$ T cell-specific epitopes. Both CD27 and CD28 expression levels have been used to classify the differentiation state of $CD8^+$ T cells and are regularly used in connection with CD45RA and CCR7 to distinguish effector and memory T cells. One study identified unique patterns of $CD8^+$ T cell differentiation in the periphery based on the specific viral infection, finding a greater frequency of $CD28^+$ virus-specific $CD8^+$ T cells from HCV-infected patients compared to HIV, CMV, or EBV; conversely, the frequency of $CD27^+$ virus-specific $CD8^+$ T cells was lower in CMV (88). The expression of CD57, meanwhile, has been linked to both $CD8^+$ T cell memory subsets (both T_{CM} and T_{EM}) but also senescent or functionally exhausted $CD8^+$ T cells, adding to the complexity of differentiating between "memory-like" vs. exhausted human $CD8^+$ T cell subsets (89, 90). In some patients, infection with *Mycobacterium tuberculosis* (TB) can result in latent infection (LTBI) where the bacteria remain quiescent until re-activation. Unlike during active TB infection, the differentiation of $CD8^+$ T cells in LTBI patients is highly skewed toward terminally differentiated effector memory cells (T_{EMRA}) as opposed to the T_{EM} compartment (91). Together, these highlighted studies demonstrate how different infections, despite their chronicity or latency, can drive a highly heterogeneous memory $CD8^+$ T cell population in patients.

An important consideration in defining T_{EX} is the co-expression of inhibitory receptors including PD-1, CTLA-4, LAG3, TIM3, 2B4, and CD160. Expression of a single inhibitory receptor is insufficient to define T_{EX}, as some inhibitory receptors such as PD-1 are upregulated upon T cell activation and therefore can also serve as activation markers. A major distinction between exhausted and memory $CD8^+$ T cells is the ability to produce cytokines such as IFN-γ, TNF-α, and IL-2 upon TCR stimulation. Terminally exhausted T cells, initially described in the chronic LCMV infection model in mice, demonstrate a marked reduction or inability to produce these cytokines upon re-stimulation, and it is this concurrent loss of polyfunctionality (the ability to produce multiple cytokines and mediate toxicity) and increasing inhibitory receptor co-expression that is crucial when defining T_{EX}. Greater insight into T cell exhaustion including molecular and cellular drivers of exhaustion is thoroughly reviewed in McLane et al. (77). In contrast, re-stimulation of memory $CD8^+$ T cells results in a high level of cytokine production that is associated with low co-expression of inhibitory receptors. Insight into T cell responses during chronic infection was provided by the observation that antigen-load plays a crucial role in the development of an exhausted $CD8^+$ T cell phenotype, as decreasing the abundance solely of GP33 (an LCMV-derived epitope recognized by $CD8^+$ T cells) on the virus while maintaining viral loads and other LCMV-derived epitopes resulted in reduced expression of PD-1 and elevated dual-production of IFN-γ and TNF-α by P14 (GP33-specific TCR transgenic) $CD8^+$ T cells (92). In support of "memory-like" $CD8^+$ T cell development during this infection is the finding that virus-specific T cells transferred into naïve mice 4 weeks after initial infection with LCMV Cl13 were able to expand and control viral titers when recipient mice were infected with the acute virus LCMV Armstrong strain, despite retaining high PD-1 expression levels and reduced (but not absent) IFN-γ and TNF-α production (93). Further, these antigen-specific $CD8^+$ T cells were maintained in the absence of antigen or infection, a foundational hallmark of memory T cells, through signaling from the homeostatic cytokines, IL-7 and IL-15, via their receptors CD127 (IL-7R) and CD122 (IL-15R). This is in contrast to an earlier finding which demonstrated that memory $CD8^+$ T cells isolated from the very late time-point of 120 days post-infection fail to persist or respond to LCMV after transfer into naïve host mice (94). Moreover, with chronic LCMV infection, long-lived virus-specific $CD8^+$ T cells during chronic LCMV infection show decreased expression of both CD127 and CD122 (95, 96). It is likely that fewer $CD8^+$ T cells present in chronically infected hosts at day 28 post-infection have yet to be driven to terminal differentiation as compared to the very late time-point of 120 days.

A hallmark of memory $CD8^+$ T cells is their capacity to proliferate upon TCR engagement, whereas T_{EX} are ultimately driven toward apoptosis. Several studies have evaluated how chronic infection affects $CD8^+$ T cell differentiation and impacts memory or "memory-like" T cell populations under these conditions. The discovery that checkpoint inhibitor blockade, notably through the use of anti-PD-1 and anti-CTLA-4 antibodies, reinvigorates exhausted T cells was a landmark finding that ultimately changed the landscape of cancer therapy. The important groundbreaking work by the pioneering studies on CTLA-4 and PD-1 by James Allison and Tasuku Honjo, respectively, was recently recognized by their receipt of the Nobel Prize in Medicine in 2018. Blockade of the PD-1/PD-L1 pathway was also found to abrogate T cell exhaustion when therapeutically administered to mice persistently infected with chronic LCMV (97, 98). While early interpretations of these data suggested the reversal of T cell exhaustion, more recent studies have identified a unique "memory-like" subset of exhausted T cells (T_{SC}) that is responsible for the T cell response

with PD-1 blockade therapy. One study has demonstrated that CD28 signaling is a cell-intrinsic requirement for LCMV-specific cells to proliferate in response to anti-PD-1 treatment (99). Further examination of PD-1$^+$ cells in LCMV Cl13-infected mice identified CXCR5 expression as a distinguishing marker of PD-1 blockade responsiveness (49). Transcriptional profiling of CXCR5$^+$ cells identified the expression of several Wnt signaling genes associated with self-renewal and hematopoietic stem cell maintenance (49). Importantly, this subset was also found to have high levels of Id3 over Id2, and high Eomes over T-bet—transcription factor profiles characteristic of memory precursor and memory CD8$^+$ T cells (49). T_{RM} also have unique transcriptional signatures from other memory T cell compartments, such as the expression patterns of transcription factors Blimp-1 and Hobit (Zfp683, "homolog of Blimp-1 in T cells") which are co-expressed in T_{RM}, with simultaneous low expression of Eomes and T-bet (86). In contrast, ZBTB32 (zinc finger and BTB domain containing 32) is another transcription factor co-expressed with Blimp-1 and limits CD8$^+$ T cell memory development during both acute and chronic viral infections (100).

Further support for "memory-like" CD8$^+$ T cell development during LCMV Cl13 infection was the identification of a role for the transcription factor T cell factor-1 (Tcf-1, encoded by the gene *Tcf7*) in a subpopulation of virus-specific CD8$^+$ T cells. Previously described as a transcription factor co-activated by β-catenin downstream of canonical Wnt signaling, Tcf-1 was found to play a role in memory CD8$^+$ T cell generation and function (101, 102). The use of Tcf-1 reporter mice identified that Tcf-1 expression in CD8$^+$ T cells was associated with the maintenance and re-expansion of virus-specific CD8$^+$ T cells in LCMV Cl13 infected mice. RNA-seq analysis of Tcf-1-expressing cells showed transcriptional characteristics that were shared with both memory and exhausted CD8$^+$ T cells, but unique from effector T cells (103). In support of their characterization as a "memory-like" T cell compartment, Tcf-1-expressing CD8$^+$ T cells show low levels of KLRG1, CX3CR1, T-bet, Blimp1, and granzymes while expressing high levels of IL-7R, CD62L, CCR7, Id3, and Bcl-6. However, Tcf-1-expressing cells share PD-1, LAG3, and c-Maf expression levels on par with exhausted T cells, supporting the concept that these cells are unique from archetypal memory cells. In humans, similar characteristics in Tcf-1-expressing cells including the ability to expand upon re-challenge stimulation were described in HCV-specific T cells (of which 20–60% were Tcf-1$^+$), demonstrating that this is not a LCMV-specific phenomenon (103). Indeed, further studies involving HCV-infected patients attribute the heterogeneity of memory CD8$^+$ T cells to differing levels of Tcf-1 expression (56). By assessing the graded expression levels of Tcf-1, a recent study found a reciprocal relationship between T-bet and Tcf-1, while Eomes expression was highest within the Tcf-1-intermediate compartment (56). While these studies have led to a greater understanding of CD8$^+$ T cell biology, most importantly they led to an important connection between Tcf-1 expression and CD8$^+$ T cell responsiveness to PD-1-targeted checkpoint blockade therapy in cancer, which is discussed below.

The vast heterogeneity of differentiated and "memory-like" CD8$^+$ T cells that arise during persistent antigen exposure demonstrates the importance in understanding the cellular and molecular drivers of protective immunity. Importantly, we must better understand the conditions that give rise to "memory-like" exhausted T_{SC} CD8$^+$ T cells, as these appear to be the cells most responsive to checkpoint blockade therapy and therefore less sensitive to terminal exhaustion. Such insights are needed to instruct the future development of new immunomodulatory checkpoint blockade therapies, establish whether a patient would be responsive to therapy, and enhance vaccination strategies.

CD4$^+$ Memory T Cell Development in Chronic Infection

The importance of CD4$^+$ T cells during persistent infections is highlighted by models in which this immune cell compartment is depleted. During chronic infection in humans and mice, decrease in helper CD4$^+$ T cells or their functional capacity is associated with less pathogen control or the establishment of chronicity (104, 105). Further, the CD4$^+$ T cell compartment plays a pivotal role by contributing to both the cellular and humoral arms of the immune response in chronic infection in both mice and humans (106). Despite the importance of the CD4$^+$ T cell response during chronic infection, greater emphasis and research has focused on understanding their role in CD8$^+$ T cell differentiation during chronic infection; much less is known about how chronic antigenic stimulation affects the differentiation and subsequent "memory-like" population of persisting CD4$^+$ T cells in the context of persistent infection.

It is a well-defined phenomenon that CD8$^+$ T cells become exhausted as a result of continuing antigenic exposure during chronic infections, as summarize above. Whether this is true for CD4$^+$ T cells remains unclear, although the development of dysfunction clearly occurs. Using the LCMV models to compare acute vs. chronic viral infections, CD4$^+$ T cells demonstrate a reduced characteristic T_H1 cell cytokine profile, i.e., reduced production of IFN-γ, TNF-α, and IL-2 (107, 108). HCV infection is also known to cause an acute infection that can progress to chronicity if not controlled. In peripheral blood, broadly reactive CD4$^+$ T cells were detected early during this infection but became undetectable in patient cohorts with chronic infection, even after viral loads diminished (109). Attempts to expand these cells *in vitro* were unsuccessful despite verification of their presence early in infection. In addition to the reduction of T_H1 associated cytokine production, many studies have also identified altered cytokine expression by CD4$^+$ T cells characterized by elevated IL-10 and IL-21 expression (107, 110–112). CD4$^+$ T cells can also develop inhibitory receptor expression patterns associated with T cell exhaustion by expressing CTLA-4, which was observed in LCMV, HIV, and HBV chronic infections; PD-1; CD160; and BTLA (108, 112–115). CD4$^+$ T cells are known to have high levels of PD-1 during chronic HIV infection and in one study this was observed to correlate with viral load (116). However, despite high levels of PD-1 expression, these cells retained the ability to produce IFN-γ (117).

Models of persistent parasite infection using *T. gondii* suggest that $CD4^+$ T cells also become exhausted, with overlapping features of Blimp-1 expression, decreased expression of co-stimulatory molecules including OX40, ICOS, and 41BB, and increased inhibitory receptor expression such as 2B4; further, a reduction in cytokine expression was observed (118). These comparisons were made based upon the graded levels of PD-1, with cells that express greater PD-1 considered to be a more exhausted phenotype (118). Persistent antigenic exposure appears to be the main driver of this phenotype as suggested by infection models with *Mycobacterium tuberculosis*, which demonstrate that the temporal availability of antigen affects cytokine expression and magnitude of the $CD4^+$ T cell response (119). Cells with limited exposure to antigens developed into cells that would be considered stereotypical memory cells, while continuously stimulated $CD4^+$ T cells have a functionally altered phenotype. Still other studies using mouse models of malaria infection showed that malaria-specific $CD4^+$ T cells exposed to chronic *Plasmodium* spp. infection had reduced cytokine production in comparison to those cells first deprived of antigen, then subsequently re-exposed in infected hosts (120, 121). Other studies have suggested exhaustion in the context of *P. chabaudi* infection, based on reduced cytokine production capacity and proliferation comparing early times points and later time points post infection (122). In humans, more evidence is needed to support this claim in *Plasmodium* infections as few studies have been performed and phenotypic analysis of $CD4^+$ T cells only suggests exhaustion by inhibitory receptor expression (123). Disregarding whether $CD4^+$ T cells are admittedly exhausted or dysfunctional, many studies in both mouse and human in multiple different chronic infections support the premise that blockade therapy of PD-1, PD-L1, or CTLA-4 augments $CD4^+$ T cell cytokine production or proliferation (113, 115).

T_H1 $CD4^+$ T cells are commonly generated as a result of both acute and persistent viral infection and can be critical for $CD8^+$ T cell function; but in the case of persistent infections, this population can be lost over time (83). For example in HIV patients, over long term treatment there is a discernable decrease in Gag293-tetramer specific $CD4^+$ T_H1 cells whereas CMV-specific $CD4^+$ T_H1 cells in the same patients remain unchanged (124). An important role for T_H1 cells in chronic infection however is highlighted by the finding that in HIV controllers, individuals who control HIV replication without antiretroviral therapy, a polyfunctional T_H1 $CD4^+$ T cell population is maintained. As a note of caution, HIV-specific $CD4^+$ T cells are often compared to CMV-specific $CD4^+$ T cells in terms of function and phenotype, despite major differences in the course of infection and viral replication kinetics. Antigen availability and viral load may also play an important role in T cell differentiation. Therefore, the characteristics of different pathogens and antigen exposure clearly play a role in T cell differentiation and function (119). A similar phenomenon was described in HCV patients, in which patients who responded to interferon-α treatment had better maintenance of a polyfunctional HCV-specific T_H1 $CD4^+$ T cell population over non-responders (125). It was shown that $CD4^+$ differentiation during chronic or prolonged antigenic stimulation in the context of infection skews $CD4^+$ T cells toward a T follicular helper (T_{FH}) cell lineage which may account for the loss of T_H1 cell cytokine production. Recent studies have therefore focused on the contribution of the T_{FH} cell population during chronic infection (126). Indeed, T_H1 and T_{FH} cells are generated early during infection with LCMV Cl-13 but the T_H1 population is not maintained (127). This enrichment of T_{FH} cells during chronic phases was also observed in SIV (simian immunodeficiency virus) models with rhesus macaques, as it was noted that chronically infected rhesus macaques had increased T_{FH} cells and this correlated with elevated IL-6 levels, the cytokine known to induce T_{FH} differentiation (128). Others had noticed a similar trend but suggested that $CD4^+$ T cell differentiation was being redirected toward a T_{FH} phenotype (129). T_{FH} cell skewing is not only observable in viral infections as patients with chronic parasitic infection, Schistosoma, show increased numbers of T cells with a T_{FH} phenotype that correlated with parasite-specific antibody levels (130). The development of a late T_{FH} phenotype was also present in the chronic phase of *Leishmania infantum* infection in rhesus macaques where there was an elevation in transcripts of *Bcl6*, *Cxcr5*, and *Il21*, all molecules associated with a T_{FH} response (131).

T helper cell differentiation is also observed during SIV infection, however new designations of "type 1 induced T_{FH} cells" have been adopted to account for those T_{FH} cells which have features of T_H1 cells, including expression of CXCR3 and IFN-γ, but are more phenotypically T_{FH} by transcription factor and surface cell marker expression (132). An interesting study in SIV-infected rhesus macaques probed the question from the opposite perspective and sought to determine the kinetics of IL-21 expression during infection (133). IL-21 was produced by multiple T_H cell subsets, but predominantly T_H1 cells and this early expression of IL-21 in T_H1 cells negatively correlated with viral load, demonstrating the importance of a polyfunctional $CD4^+$ T cell response in the early stages of a chronic infection (133). Variability in cytokine expression of $CD4^+$ T cells suggests that the initial classification of $CD4^+$ T cells into subsets based on cytokine production and transcription factor expression will likely need to be revisited in the context of chronic antigen stimulation in infections. What is unclear is whether persisting T_{FH} exhibit features of effector, memory, or exhausted T cells. As RNA-seq becomes more widely used as well as the ability to obtain transcriptomes of fewer cells using single-cell RNA-sequencing (scRNA-seq), the degree of heterogeneity in the $CD4^+$ T cell population is becoming more apparent and will likely lead to new insights into $CD4^+$ T cell responses to chronic antigen stimulation (112, 134).

At present, with the observation that there may be skewing of $CD4^+$ T cell subsets during chronic or prolonged antigenic exposure, there are a few explanations as to the mechanism by which this occurs. Due to constant replication, studies with LCMV suggest that the exposure to type I IFN inhibits the *de novo* T_H1 differentiation; this was first only surmised to be an indirect effect on $CD4^+$ T cells as IFN receptor deficient $CD4^+$ T cells did not augment the number of T_H1 cells (127). Later experiments would support this claim, demonstrating that type I IFN induced IL-10 and PD-L1 on dendritic cells that

would then suppress T_H1 differentiation, and the subsequent loss of T_H1 help would contribute to $CD8^+$ T cell dysfunction (83). T_{FH} cell differentiation is likely driven by IL-6 that is produced later during the course of chronic LCMV infection (135). Recent studies in mice lacking the TCR scaffolding protein CD2AP, thus resulting in altered TCR signal strength, demonstrated increased T_{FH} generation and a concomitant increase in neutralizing antibody activity in LCMV which implies a role for TCR signaling in T_{FH} generation during infection (136).

Glucocorticoid-induced tumor necrosis factor related protein (GITR) is another molecule that was demonstrated to be important for $CD4^+$ T cell differentiation during chronic LCMV infection (137). GITR-deficiency was shown to inhibit $CD4^+$ T cells in the early T_H1 production of IL-2 that is needed to support $CD8^+$ T cell proliferation as well as the late T_{FH} cell response to promote humoral immunity through provision of B cell help. Thus, it remains possible that T_H1 and T_{FH} are both generated during the initial infection and T_H1 cells are not maintained during chronic infection. This differentiation toward a sustained T_{FH} cell presence during chronic infection appears to provide many benefits to the immune response. T_{FH} are named for their role in providing help to B cells and orchestrating the germinal center reaction (138). Importantly, resolution of chronic viral infection with LCMV is dependent on antibody production promoted by T_{FH} cells (139, 140). The importance of T_{FH} in HIV is also well-noted as the number of these circulating cells positively correlated with the presence of broadly neutralizing antibodies (141). During chronic or prolonged infections, many have observed the production of IL-21 by additional $CD4^+$ T cell subsets including T_{FH} and T_H17 cells (112, 142). Although typically associated with its importance in the germinal center reaction, in the context of chronic or prolonged infection, this cytokine has been shown to support $CD8^+$ T cell function. Early studies in the LCMV chronic infection model noted the importance of $CD4^+$ help to $CD8^+$ T cells in the form of IL-21, however this appeared to come at a cost of reduced T_H1 cytokine production in $CD4^+$ T cells (143).

In the LCMV model, IL-21 signaling was linked to the induction of the transcription factor BATF in $CD8^+$ T cells, which is important for maintenance of $CD8^+$ T cell effector function (106). Similar evidence for IL-21 production preventing $CD8^+$ T cell exhaustion during chronic infection was observed in a mouse model of parasitic infection using *T. gondii* (144). Beyond its role in the $CD8^+$ T cell response, IL-21 deficiency was also observed to compromise the humoral arm in *T.gondii* infections, leaving mice more susceptible to toxoplasmic encephalitis (145). Lack of IL-21 signaling by global deletion of the IL-21 receptor (IL-21R) brought about increased inhibitory receptor expression on $CD8^+$ T cells concomitant with greater parasite burden and reactivation (144). This susceptibility due to IL-21 insensitivity was also observed in a mouse model of tuberculosis (146, 147). When considering HIV in humans, small populations of IL-21-producing $CD4^+$ T cells were present in the blood of patients with acute and chronic HIV and a greater frequency of HIV-specific $CD8^+$ T cells expressed the IL-21R when compared to CMV-specific T cells (148). Combined with data suggesting that IL-21 ligation of IL-21R on HIV-specific $CD8^+$ T cells enhanced effector molecule production, these findings support the role of $CD4^+$ T cell derived IL-21 in providing necessary help to sustain $CD8^+$ T cells during chronic infection (149). In studies of HIV/HCV co-infected individuals, these IL-21 producing $CD4^+$ T cells were also associated with viral control, further supporting the role of this cytokine in antiviral immunity (150). Although attributed to T_H17 cells, in SIV infection of rhesus macaques, IL-21 supported $CD8^+$ T cell responses and prevented exhaustion (151).

Compared to $CD8^+$ T cells, more information on $CD4^+$ T cell differentiation during chronic infection is needed to accurately determine what effect chronic antigenic stimulation has on T helper cell differentiation and function. Whether T_{FH} or IL-21-producing $CD4^+$ T cells that persist with time after chronic infection form "memory-like" cells has yet to be studied. Of note, this review does not discuss the implications chronic antigenic stimulation has on the development or differentiation of regulatory $CD4^+$ T cells, or the levels of inhibitory receptor expression and suppressive cytokine production expressed by these cells. Many of the studies discussed, however, highlight the plasticity and heterogeneity present within the helper $CD4^+$ T cell population as an adaptive immune cell that appears to be dynamically regulated by temporal and environmental dimensions. As noted above, future transcriptome studies utilizing scRNA-seq will enable further insight into the regulators that determine $CD4^+$ T cell fate during chronic infection but also the profile of these cells. These studies can also help answer the question of whether the different antigen specific CD4+ T cell subpopulations are selectively lost as a result of chronic infection or their differentiation is skewed toward alternative differentiation lineages as the "memory-like" compartment develops. More recent studies have already hinted at the limitations of staining for a few markers and the possibility that $CD4^+$ populations are much more polyfunctional than previously anticipated (133). This polyfunctionality of $CD4^+$ T cell subsets, as demonstrated the ability of different cells to contribute to both humoral and cellular immunity (e.g., T_H1 and T_{FH}), highlights the importance of the different $CD4^+$ T cell compartments and warrants further research to understand the dynamics and differentiation during chronic infections, and whether "memory-like" $CD4^+$ T cells contribute to the sustained responses to chronic infections.

T CELL RESPONSES TO CANCER AND CANCER-ASSOCIATED ANTIGENS

Although our knowledge of effector, memory, and exhausted T cell differentiation largely comes from studies using virus and other infection models, it is crucial to better understand the extent of memory T cell formation in response to tumors as this can instruct the development of novel cancer treatments and aid in the development of vaccine strategies against cancer, particularly as recent studies have similarities between T cell subsets derived from chronic infection and tumors (152).

From studies in both mice and humans, it is becoming more appreciated that the efficacy of anti-tumor responses is enhanced by the generation of both $CD4^+$ and $CD8^+$ "memory-like" T cell compartments.

$CD8^+$ Memory T Cell Development in Tumors

The priming of tumor-specific $CD8^+$ T cells occurs in the lymph nodes by DCs that take up and cross-present neoantigens from the tumor, and activated tumor-specific T cells migrate into tumors guided by cytokine gradients (153, 154). Highly cytotoxic tumor-specific effector $CD8^+$ T cells are a fundamental component of protective tumor infiltrating lymphocytes (TIL), and strongly correlate with patient survival (155, 156). After tumorigenesis, as with chronic infections, tumor-specific $CD8^+$ T cells can become progressively dysfunctional and further persistence of the tumor can ultimately lead to the establishment of a permanent state of exhaustion (157, 158). At this stage, exhaustion cannot be reversed by anti-PD-1 therapy due to epigenetic modifications that prevent transcription of genes associated with effector function (158). As found with chronic virus infections, a major defining characteristic of T_{EX} in tumors is the increased expression and co-expression of multiple inhibitory receptors that include PD-1, Tim3, LAG3, CD160, and TIGIT, the absence of the transcription factor Tcf-1 with high expression of TOX, and progressive reduction in effector functions that are linked in part to dysregulated metabolism (77, 159). The critical role of TOX in the development of $CD8^+$ T_{EX} in both chronic virus infections and cancer has only recently been described, with several studies identifying the necessity for TOX in T_{EX} development. These studies show a role for TOX in regulating chromatin accessibility/epigenetic modifications associated with T_{EX}, and its expression is driven by NFAT and chronic TCR stimulation (70–73). However, dysfunctional tumor-specific $CD8^+$ T cells can display two different chromatin states: a plastic and fixed dysfunctional state (160). Those cells within the fixed dysfunctional chromatin state are resistant to reprogramming and express high levels of CD38 and CD101, whereas PD-1^+ TIL lacking CD38 and CD101 can undergo reprogramming to develop into effector cells (160).

Alterations in surface marker expression are determined by the transcriptional profiles of tumor-specific $CD8^+$ T cells that define the differentiation states of the cells including "memory-like" $CD8^+$ T cell compartments. Transcriptome analysis of tumor-specific $CD8^+$ T cells from non-small cell lung carcinoma (NSCLC) and melanoma patients has identified the altered expression patterns of several transcription factors known to be major regulators of effector and memory $CD8^+$ T cell differentiation, including Blimp1, Id2, T-bet, and Eomes (65, 161, 162). Phenotypically, in addition to expression of various inhibitory receptors, PD-1^{hi} $CD44^{int}$ $Eomes^{hi}$ $CD8^+$ T cells exhibited a terminal T_{EX} cell phenotype, whereas PD-1^{low} $CD44^{hi}$ $Eomes^{lo}$ T-bet^{hi} $CD8^+$ T cells could form effector cells. Terminal T_{EX} cells are characterized by high expression of Eomes and decreased levels of T-bet. First defined in chronic LCMV infection, PD-$1^+$$CXCR5^+$$Tim3^-$ $CD8^+$ T cells in the lymphoid organs were found to be responsive to PD-1 blockade therapy and express the transcription factor Tcf-1 while sharing a common gene signature with $CD8^+$ memory precursors and were subsequently denoted as T_{SC} (49). Similar to virus-specific T_{SC}, intratumoral melanoma tumor-antigen-specific Tcf-1^+PD-$1^+$$CD8^+$ T cells exhibit stem-like properties that include self-renewal and proliferation and expanded in response to checkpoint blockade were found to have characteristics of both T_{EX} and T_{SC} (163). In melanoma patients, the Tcf-1^+PD-$1^+$$CD8^+$ T cell population increased in response to anti-CTLA-4 and/or anti-PD-1 treatment and there is the potential that detection of this population can predict patient survival. CX3CR1 expression is also associated with increased responsiveness to PD-1 checkpoint blockade therapy, as increased expression of CX3CR1 on $CD11a^+$$CD8^+$ T cells in NSCLC patients strongly correlated with a positive clinical response to treatment (164).

Because of the recognized heterogeneity of $CD8^+$ T cells within tumors, the use of single-cell analysis techniques is yielding important new insights into the unique properties of tumor-specific T cells. A recent study from Sade-Feldman et al. used scRNA-seq to address whether patterns in the tumor transcriptome could predict patient responses to checkpoint blockade therapy (162). In comparing the transcriptomes of tumors from 48 melanoma patients, their study highlighted the heterogeneity of the $CD8^+$ T cell compartment and identified a strong correlation between the expression of Tcf-1 in $CD8^+$ T cells and clinical responses to checkpoint blockade (162). Although they did not detect an association with CXCR5 expression and T cell responsiveness in their patient population as was found in previous studies, they showed that expression of CD39 was indicative of $CD8^+$ T_{EX} cells. Several recent studies have also shown that TILs are a highly diverse T cell pool. Li et al. found that $CD8^+$ TILs from melanoma patients form a gradient of dysfunction as indicated by transcription factor and inhibitory receptor expression (165). Furthermore, dysfunctional $CD8^+$ T cells maintained the ability to clonally expand in the early phase of tumor progression (165). In a study of hepatocellular carcinoma (HCC), scRNA-seq analysis not only highlighted an enrichment of $CD8^+$ T_{EX} in HCC, but also identified a CX3CR1 cluster of effector "memory-like" $CD8^+$ T cells, drawing parallels to the findings in NSCLC (166). At this junction, it does appear that these cells, which are only found in the context of chronic antigen stimulation, can be considered to be memory cells despite some overlap in gene signatures with T_{EX}.

In both humans and mice, there is evidence supporting the development of tumor-specific "memory-like" $CD8^+$ T cells, which may be favored at the early stages of tumor growth when the extent of inflammation and levels of antigen exposure are reduced compared to later stages of cancer progression. In melanoma patients that received adoptive T cell therapy, it was shown that the infused $CD8^+$ T cells developed a T_{CM} phenotype *in vivo* (167). Further, in some patients with colorectal cancer, T_{EM}- and T_{CM}-like populations have been identified, demonstrating the possibility that memory $CD8^+$ T cells may naturally develop in response to cancer antigens. In one study, $CD8^+$$CD45RO^+$$CCR7^-$$CD28^+$$CD27^+$ effector memory phenotype T cells were detected within colorectal tumor

resections and were associated with increased survival in patients and noted a positive correlation between the infiltration of "memory-like" $CD8^+$ T cells and patient survival (168). In particular, high levels of "memory-like" $CD45RO^+$ cells within the tumor strongly correlated with the absence of early metastatic disease. In breast cancer patients, it has been shown that the ratio of the "memory" T cell compartment ($CD45RO^+$) compared to naïve T cells in the bone marrow was significantly increased for both $CD4^+$ and $CD8^+$ T cells in patients compared to healthy controls, with the greatest increase in memory phenotype $CD4^+$ T cells in bone marrow of patients where disseminated tumor cells were detected. Although the significance of these findings is unclear, this study found that despite an initial increase in HLA-A2/Her-2/$neu_{369-377}$ tetramer-binding tumor specific "memory-like" T cells in the bone marrow, as the tumor advanced to later stages, this population ultimately decreased (169). This could potentially indicate a role for antigen load in the deletion or distribution of "memory-like" tumor-specific T cells. From mouse studies, it is thought that T_{CM} may be more protective and effective against cancer compared to T_{EM}, in part due to their high levels of IL-2 production and capacity for proliferation (170). One study found that, on a per-cell basis, *in vitro*-generated tumor-specific T_{CM}-like $CD8^+$ T cells were able to mount a strong recall response to tumors greater than that of their T_{EM}-like cultured counterparts and were capable of eradicating established tumors when combined with both exogenous IL-2 and a cancer-antigen vaccination strategy (170).

Vaccination strategies have also been employed to promote the development of tumor-specific memory $CD8^+$ T cells, recognizing the importance of these T cells in achieving long-term tumor control. In one very promising study in mice, vaccination was applied after tumor excision. Following excision of primary B16 melanoma tumors, mice were vaccinated with optTRP1_{455} peptide and also given TGF-β blockade to reverse the tumor and regulatory $CD4^+$ (T_{reg}) cell TGF-β-mediated suppression of $CD8^+$ T cells (171). Strikingly, upon re-challenge with B16 tumors, mice that had received both treatments showed increased protection with 50% of mice failing to develop tumors. This was attributed to the development of a protective $CD8^+$ T cell population characterized by the stronger prevalence of tumor-infiltrating $CD8^+$ T cells with a memory precursor phenotype. Another study aimed at exploring the impact of T_{regs} on limiting the development of effective $CD8^+$ T cell responses to B16 melanoma. This study found that prophylactic depletion of T_{regs} by anti-CD25 treatment prior to primary tumor engraftment and followed by primary tumor resection resulted in protection of 80% of mice against secondary tumor growth re-challenge (172). Further, deletion of the bulk $CD4^+$ T cell population allowed for long-lived antigen-specific $CD8^+$ T cells in secondary lymphoid organs and were protective after primary tumor resection against both localized and systemic secondary tumor challenges. While these studies demonstrate the potential for "memory-like" $CD8^+$ T cell formation in response to tumors, it is unclear how long these "memory-like" populations persist in patients and their efficacy in protecting against relapse. It is equally important to recognize that these populations may only arise in cancers that are more localized (e.g., breast cancer, melanoma) rather than systemic (e.g., leukemia or lymphoma) and that the rate of disease progression may play a major role in determining if "memory-like" $CD8^+$ T cells will form.

Recently, there has been great interest in the T_{RM} compartment in cancer due to their function in local protection against repeat infections (173). Previous studies have shown that human lung tumor-infiltrating $CD8^+$ T cells express high levels of CD103 and CD69, and low levels of CD62L and CCR7, suggestive of T_{RM} which retain characteristics of activated cells and induce rapid and effective responses against disease (174). In the tumor microenvironment, abundant TGF-β and T cell receptor signaling through the tumor antigen/MHC class I (MHC-I) complex has been shown to induce the formation of tumor-specific $CD8^+CD103^+$ T cells (175). TGF-β signaling triggers CD103 expression on T cells, and enhances the lytic function of anti-tumor $CD8^+$ T cells (176). T_{RM} cells in human lung cancers express high levels of granzyme B, perforin, CD107a, and IFN-γ (177). Further, CD103 interactions with E-cadherin induces CCR5-mediated recruitment of $CD8^+$ T cells into tumor as well as polarization and exocytosis of cytolytic granules, ultimately leading to tumor cell lysis (178). Tumor-infiltrating cells with a T_{RM} phenotype from advanced melanoma and lung cancer patients express higher inhibitory receptors such as PD-1, Tim3, and LAG3, which opens up the possibility that checkpoint blockade might promote the greater anti-tumor immunity by T_{RM} cells (177, 179, 180). Studies in mice have provided encouraging evidence for the ability of PD-1 blockade therapy to promote the infiltration of T_{RM}-like ($CD69^+CD103^{+/-}$) $CD8^+$ OT-I T cells generated from transferred vaccination-derived T_{CM} ($CD44^+CD62L^+$) into both B16-OVA and MC38-OVA (181). In both model systems, the addition of PD-1 blockade resulted in better tumor control and increased numbers of T_{RM}-like donor OT-I cells per gram of tumor. As the prevalence of checkpoint blockade therapy in patients grows, it will be important to evaluate how these therapies contribute to the development of "memory-like" $CD8^+$ T cells in patients that are in remission. Losing the potentially beneficial contribution of TGF-β to T_{RM} formation in the tumor microenvironment must therefore be considered when thinking about therapeutic TGF-β to limit $CD8^+$ T cell inhibition.

At the molecular level, T_{RM} cells do not express Eomes and Tcf-1, which are expressed by other memory T cell subsets [**Table 1**, (182, 183)]. Absence of Eomes expression is required for CD103 induction and low expression of T-bet is necessary for expression of CD122 and maintaining IL-15 responsiveness by T_{RM} cells (175). On the other hand, expression of the transcription factors Hobit (homolog of Blimp1 in T cells) and Blimp1 promote the retention of T_{RM} cells in multiple organs and suppress genes related to egress from tissues (86). Runx3 is required to form T_{RM} cells in various tissues and tumors (62), and the transcription factors BATF (which is essential in the differentiation of effector T cells) and NAB1 (which is proposed to prevent apoptosis of TILs) are also upregulated in T_{RM} cells in tumors (177). Although the function of T_{RM} cells in anti-tumor immunity has not yet been fully addressed, accumulating data indicates that the cells can have a crucial

role in anti-tumor responses (30). Malik et al. showed that skin-resident T_{RM} induced by vitiligo have a $CD103^+CD69^+$ phenotype and are beneficial in protecting against melanoma (126). In untreated lung cancer patients, the density of $CD103^+$ T_{RM} cells among tumor-infiltrating $CD8^+$ T cells shows a high potential as a prognostic markers for increased patient survival (177). Similarly, $CD103^+$ TILs from high-grade serous ovarian cancer (HGSC) correlate with better patient survival (184).

Taken together, the studies of $CD8^+$ T cells in anti-tumor responses support the possibility of generating *bona fide* tumor-specific memory particularly in the context of localized tumors and as a consequence of vaccination strategies with tumor-specific epitopes that can be generated by cancers with frequent mutations. Moreover, with adoptive cell therapies such as those based on TILs, it may ultimately be possible to preselect memory cells to develop infusion products that can become established as memory cells and thereafter maintained to protect against re-emergence of tumors such as observed with the persistence of the chimeric antigen receptor (CAR) T cell therapies (185).

$CD4^+$ T Cell Memory Development in Tumors

While the main focus of basic and clinical research has been on improving $CD8^+$ T cell-mediated eradication of tumor cells, the role of $CD4^+$ T cells in tumor immunotherapy is much less developed. Moreover, evidence for the involvement of $CD4^+$ T cells in tumor eradication extends beyond the canonical function of helper T cells and their ability to promote $CD8^+$ T cell and B cell responses. These include direct effects on tumor cells by cytokines produced by $CD4^+$ T cells such as IFN-γ, TNF-α, and IL-2, modulation of DCs and other antigen presenting cells in the tumor microenvironment as well as direct killing of tumor cells by cytolytic $CD4^+$ cells. As such, promoting $CD4^+$ responses to tumors and the generation of $CD4^+$ T cell memory are crucial to developing an effective anti-tumor immune response.

It has been known for some time that MHC class II-restricted (MHC-II) tumor antigens were capable of initiating $CD4^+$ T cell responses critical for maintenance of anti-tumor immunity (186). More recently, MHC II-restricted neoantigens were found to possibly be more effective targets for cancer immunotherapy (187). Using these neoantigens in tumor targeted vaccine-based strategies is thus an important consideration for promoting memory development. In certain tumors such as breast cancer, the presence of memory phenotype T cells are a prognostic indicator for anti-tumor responses, with an increase in T_{CM}-like and decrease in T_{EM}-like $CD4^+$ cells in the lymph nodes of patients progressing from stage I to stage III disease (188). Similarly, an increase in intratumoral $CD4^+$ T_{EM} in colorectal tumors correlated with disease-free and survival rates in patients (155, 189). In the case of immune checkpoint blockade therapy, it was recently shown that an increase in a subset of central "memory-like" ($CD27^+Fas^-CD45RA^-CCR7^+$) $CD4^+$ T cells in patients with malignant melanoma could be used as a predictor of clinical response to PD-1 blockade therapy (190, 191). In fact, $CD4^+$ T cell memory could be induced by tri-specific antibody treatment targeting immune checkpoint inhibitors to the tumor and activating tumor-specific both $CD4^+$ and $CD8^+$ T cells simultaneously, with the greatest effect observed in the $CD4^+$ T_{EM} and T_{CM} compartments in mice (192). Thus, there is great therapeutic potential in harnessing the power of memory $CD4^+$ T cells to promote the most effective anti-tumor immune responses.

Although cytotoxic $CD8^+$ T cells have been the focus of eliciting an anti-tumor response, it is clear that this response benefits from $CD4^+$ T cell help and it has been shown that cross-priming of $CD8^+$ T cells by DCs requires $CD4^+$ T cell help for effective cytotoxic $CD8^+$ T cell responses (193–195). DCs involved in the initiation of the anti-tumor T cell response also benefit from $CD4^+$ T cell help, as CD40/CD40L interaction with $CD4^+$ T cells is required to fully activate DCs that can subsequently generate $CD8^+$ T_{EFF} and long-lasting $CD8^+$ T cell memory (196). Further, it has been shown that T_H1 cells can induce cytotoxic DCs that can kill tumor cells (197). Conversely, inhibition MHC-II antigen presentation by DCs to $CD4^+$ T cells also promotes the development of anergic anti-tumor $CD8^+$ T cells (198). PD-1^+ tumor-specific $CD8^+$ T cells are found in the blood of melanoma patients, indicating that priming of these T cells has occurred, although these cells are largely dysfunctional and resemble T_{EX} cells that develop during chronic infections (158, 199). Interestingly, these T_{EX} cells also are very similar to T cells which have not received $CD4^+$ T cell help, suggesting that the tumor specific $CD8^+$ T cells identified following initial priming by DCs did not see $CD4^+$ T cell help at that time. These cytotoxic $CD8^+$ T cells have been shown to be excluded from the tumor microenvironment in part due to TGF-β signaling (200). This exclusion is associated with poor clinical outcome as well as poor response to immune checkpoint blockade therapy (155, 201). Moreover, $CD4^+$ help during priming can provide the signals needed to promote invasiveness of cytotoxic $CD8^+$ T cells (202, 203). In addition, polyclonal $CD4^+$ T cells from MHC-II-negative ovarian cancer tumor-bearing mice were able to secrete CCL5 and recruit $CCR5^+$ DCs to the tumor (204). This was also shown to be important to optimize $CD4^+$ T cell help to cytotoxic $CD8^+$ cells as CCR5 ligands can improve the anti-tumor response (205, 206). Although some tumor cells do not express MHC-II, it has previously been shown that $CD4^+$ T cells can still mediate rejection of these MHC-II-deficient tumors through indirect mechanisms and there is also evidence for the development of a $CD4^+$ T cell anti-tumor memory compartment in breast cancer patients and in the B16 melanoma mouse model (198, 207–209). In breast cancer patients, analysis of bone marrow detected both T_{CM} and T_{EM} phenotype $CD4^+$ T cells, and the adoptive transfer of these cells into NOD scid mice with patient tumor transplants showed infiltration of these cells into the tumors (210). This suggests that $CD4^+$ T cell help promotes CTL responses through the recruitment of functional $CD8^+$ T cells primed by DCs and capable of migrating into the tumor. In a Her2-positive breast cancer model in mice, one study found that bulk "memory" $CD4^+$ T cells from viral immune-oncotherapy cured tumor-bearing mice proliferated upon either *in vivo* or *in vitro* challenge (211). In B16 melanoma, administration of DCs loaded with apoptotic B16 cells to mice promoted the development of a long-lived functional anti-tumor $CD4^+$

T cell compartment that produced IFN-γ upon stimulation. Importantly, this compartment was highly protective as mice subsequently challenged with B16 tumors were protected unless $CD4^+$ (or $CD8^+$) T cells were depleted prior to tumor challenge (212). Taken together, these studies demonstrate that generating the formation of a long-lived, functional "memory-like" $CD4^+$ T cell compartment can provide anti-tumor immunity. In addition, the long-lived and highly proliferative population resembling T_{SC} cells can be generated *in vitro* by activating $CD4^+$ T cells by co-culture with stromal cells expressing Notch ligands (213). Importantly, these cells can expand and develop into tumor-specific effector cells after restimulation, a promising prospect for adoptive cell immunotherapy.

Thus far, the development of cancer vaccines solely focusing on $CD8^+$ T cell epitopes has not been particularly successful without considering $CD4^+$ T cell help (214, 215). Immune adjuvant therapy, the administration of an immune stimulant in connection with treatment, has been found to be beneficial in generating anti-tumor immunity by promoting T cell memory (216, 217). As an example, in breast cancer patients, peptide vaccination using the E75 peptide in combination with GM-CSF in breast cancer patients was able to activate both naïve $CD4^+$ T cells as well as memory-phenotype $CD4^+$ T cells specific for the tumor. Sustained anti-tumor $CD4^+$ T cell "memory-like" formation was also shown in a vaccine trial of prostate cancer patients utilizing the AE37 vaccine and the DR11/AE37 tetramer to identify AE37 specific T cells. AE37 specific $CD4^+$ T cells were detected up to 4 years following vaccination, and retained responsiveness as shown by peptide stimulation (218). Work by Bergman et al. has shown the effectiveness of generating potent anti-tumor $CD4^+$ memory response (211, 219). These studies utilized viral oncolytic immunotherapy to prime T cell responses that were otherwise suppressed by chemotherapy-based regimens. Memory recall capability was shown by adoptive cell therapy and while transferred $CD8^+$ T cells were poor in controlling tumor growth, transfer of memory $CD4^+$ T cells was capable of resolving established tumors, albeit when injected in high numbers. Therefore, any consideration of adoptive immune cell therapy or cancer vaccines should include promoting the development of antigen-specific memory $CD4^+$ T cells.

Even though providing help is a major role for $CD4^+$ T cells in anti-tumor immune responses, $CD4^+$ T cells can contribute directly to regulation of the tumor microenvironment and to killing of cancer cells (220, 221). It was suggested that $CD4^+$ cells kill tumor cells through a mechanism that did not involve Fas/FasL or TNF-α, but was dependent on the TNF-α related apoptosis inducing ligand (TRAIL) (222). T_H1 $CD4^+$ T cell responses can support anti-tumor immunity, in part due to the direct impact IFN-γ has on tumor cells (194). One study described T_{EM} $CD4^+$ T cells that were capable of tumor elimination and this was dependent on IFN-γ (223). Strikingly, tumor reactive cytotoxic $CD4^+$ T cells could be induced following checkpoint blockade therapy (224). These $CD4^+$ T cells expressed Eomes but not T-bet, secreted IFN-γ, expressed granzyme B and perforin, and were capable of lysing autologous tumor cells (224). Similarly, it was shown that OX40 engagement induced both cytotoxic and memory $CD4^+$ T cells characterized by Eomes expression (221). These cells were capable of controlling tumors in mice and lysing human tumor cells *in vitro*. Thus, independent of their function in providing help, $CD4^+$ T cells can be generated that can directly target cancer cells for elimination.

Taken together, these studies in both humans and mice identify not only the potential for memory anti-tumor $CD4^+$ and $CD8^+$ T cell development, but also highlight their strong anti-tumor potential. Moreover, developing new strategies aimed at generating optimal $CD4^+$ T cell responses and memory in the context of chronic antigen exposure may offer treatments for cancers that are resistant to current immunotherapies.

DISCUSSION

The many studies discussed within this review demonstrate the possibility of memory $CD4^+$ and $CD8^+$ T cell generation under conditions of chronic or persistent antigenic stimulation. Perhaps most importantly, they highlight the high degree of diversity and heterogeneity of long-lasting and persisting memory or "memory-like" T cells generated in patients. The importance of this diversity is shown by the reproducible formation of highly heterogeneous memory T cells within genetically identical mice and with TCR transgenic T cell models where the T cell repertoire is defined (225). Memory T cell diversity, in part, reflects an array of persisting antigen-experienced T cells that have progressed through various stages of differentiation in different contexts of antigen exposure in different tissues. Indeed, a process of tissue "imprinting" can govern the migration and maintenance of memory T cells in sites such as the gut associated tissues, skin, and lung. A major contribution to memory T cell fate determination is the antigen dose and extent of the inflammatory milieu, which can drive the development of terminal effectors that are lost during the contraction phase as antigen becomes cleared (226). Indeed, exposure of T cells to lower levels of antigen at this stage of a response can favor the generation of memory cells with the capacity for self-renewal (227). A robust immune response and rapid pathogen clearance by the T cell response favors greater generation of such memory T cells, and it is cells with similar properties (e.g., Tcf-1 expression) that can respond to immune checkpoint blockade in the settings of chronic antigen exposure (49, 228). These observations underscore the concept that antigen-experienced memory T cells that retain functional and protective capabilities are generated during chronic exposure to antigens but are unable to respond because of suppressive mechanisms in the local environment. Factors like impaired antigen-presentation, limited T cell activation in response to TCR signaling, and metabolic suppression that impair to differentiate into secondary effectors and elicit control of chronic infections and cancers also inhibit the generation of memory T cells. Although we have identified some of the parameters that distinguish subsets of memory T cells and are beginning to clinically exploit properties that promote their function, it is clear that identifying strategies that promote the development of memory in the context of chronic antigen-exposure will be crucial.

Many publications also highlight the perhaps long-standing misconception that $CD4^+$ and $CD8^+$ T cells follow similar differentiation pathways or develop similar characteristics as a result of chronic antigenic stimulation. This may very well be a result of differences in peptide-stimulation itself, as $CD8^+$ T cells encounter peptide on nearly all nucleated cells in the context of MHC-I while $CD4^+$ T cells are somewhat more protected from this constant bombardment of antigen by the more restricted expression of MHC-II. Indeed, many autologous T cell transfer strategies aim to expand T cells *ex vivo* and in turn provide them with a period of antigen deprivation whereby T cells can be rested from these debilitating environments. These models also highlight not only the effect that the degree of antigen exposure has on T cell development, but also introduce a temporal aspect. Dysfunction is favored by longer duration of exposure to persistent antigen-stimulating environments that decreases the likelihood of "rescuing" these cells from dysfunctional differentiation states. Limiting antigen or reducing the time of exposure may reveal key aspects to direct future avenues for restoring the proper differentiation pathway of T cells exposed to chronic antigenic stimulation.

Another concept not fully discussed within this review is that of memory inflation, or the temporal increase in a T cell population with a virus-specific (tetramer$^+$) "effector-memory" phenotype ($CCR7^{low}CD62L^{low}CD28^{low}CD27^{low}$) and accumulation of these cells in many non-lymphoid tissues. First defined in mouse models of murine CMV (MCMV), memory inflation has also been observed humans following CMV infection, parvoviruses B19 and PARV4, chronic norovirus, extreme responses to EBV, and to adenovirus-based vaccinations (229). It is currently understood that antigenic persistence is a requirement for memory T cell inflation and is believed to be driven by sites of latent virus infection, as removal of the primary site of viral replication (e.g., the salivary glands) does not stop the phenomenon of memory inflation (230). An important distinction however between "classical" T_{EX} formed under persistent antigenic conditions such as with LCMV Cl13 or HIV infections compared to T cells generated via memory inflation is the retention of effector cytokine production and an overall lack of T_{EX} hallmark features such as co-expression of inhibitory receptors. The localization of inflationary memory T cells in non-lymphoid peripheral tissues is a hallmark they share with T_{RM}; however, while T_{RM} are confined to the tissue in which they were generated, a high number of inflationary memory T cells can be found in circulation after MCMV and CMV infection (229). Transcriptional profiling of both inflationary T cells and T_{RM} identified some commonalities between the two T cell types (e.g., upregulation of chemokine receptors and T-bet), but also showed significant transcriptional diversity (e.g., upregulation of AP-1 family members in T_{RM}, and IRF8 and EZH2 in inflationary T cells) (231). This distinction further highlights the high potential for diversity in T cell differentiation and stresses the importance of understanding how antigen availability and persistence can influence the development of functional memory or "memory-like" T cells compared to exhaustion.

Evaluation of the contribution of $CD4^+$ T cells is often neglected as $CD8^+$ T cells have a more direct role in cell elimination; however, $CD4^+$ T cells are important for both cellular and humoral immunity. As cells supporting both arms of immunity, they warrant further study into the roles they play during persistent antigen exposure and what affect they could have on promoting memory T cell formation. A loss of $CD4^+$ T cell help contributes to $CD8^+$ T cell dysfunction in chronic viral infection, but the potential for $CD4^+$ T cells to form memory during chronic infection remains unconfirmed. In addition, more studies into the direct effects of $CD4^+$ T cells on the tumor microenvironment and their contribution to the killing of cancer cells are needed. An important question that remains unanswered is whether the presence of a memory $CD4^+$ T cell pool limits the establishment of secondary/metastatic tumors by affecting the tumor microenvironment. And further, to what extent is the maintenance $CD4^+$ T cell help required to sustain the cytotoxic effector functions of $CD8^+$ T cells within tumors? Further studies on $CD8^+$ T cells are also needed to define parameters that limit $CD8^+$ T cells development into true memory compartments, to address how dysfunctional differentiation pathways can be skewed toward successful memory, and to identify possible interventions to establish functional memory. As sequencing techniques have become more robust and with the advent of methods that allow for RNA transcriptome analysis to be performed on smaller cell numbers, a large emphasis has been placed on understanding the molecular determinants of memory T cell formation. Perhaps the greatest aide in understanding the complex and heterogeneous memory T cell pool has been the development of scRNA-seq, as this allows for the first time the evaluation of transcription factor co-expression and relative expression levels on the single-cell level. It also raises the question as to how different subsets arise from the same inflammatory environment and antigenic stimuli.

Not fully discussed in this review are the important findings regarding changes in epigenetics and their contributing role in T cell differentiation and particularly dysfunction, as these have been extensively and recently reviewed elsewhere (77). It is clear that changes in DNA methylation and chromatin structure play an important role in both $CD4^+$ and $CD8^+$ T cell fate decisions, and studies aimed at deciphering patterns in epigenetic remodeling of T cells during chronic infection and cancer have provided key insight into the regulation of T cells that are effective in killing infected or malignant cells (232, 233). Future studies combining evaluation of memory and exhausted T cells that arise during chronic antigen stimulation at both the epigenetic and transcriptome level may provide key insight into targets for therapies that promote the formation of beneficial T cell responses.

Greater consideration is now being given to the influence of metabolism on T cell differentiation and memory T cell development, particularly under the conditions of chronic or persistent antigen. Several groups have now demonstrated the unique metabolic requirements of the different T cell subsets, such as the glycolytic switch that occurs upon TCR stimulation and the subsequent switch back to fatty acid oxidation (FAO) and oxidative phosphorylation (OXPHOS) by *bona fide* memory T cells (234, 235). In chronic virus infections, it has already been demonstrated that exhausted T cells develop altered metabolism

compared to functional virus-specific T cells, specifically an increased reliance on glycolysis and inability to use oxidative phosphorylation when exhausted (236). This may be a crucial distinction, since in the case of cancer, tumor cells can outcompete T cells for glucose (237). More recently, it has been shown that checkpoint blockade therapy can affect T cell metabolism, as both PD-1 and CTLA-4 signaling have been shown to inhibit glycolysis and PD-1 signaling promotes FAO in T cells (238, 239). As glycolysis has previously been linked to the production of inflammatory cytokines by T cells (240), this is an important consideration when trying to reverse T cell exhaustion in patients and promote memory T cell development as memory T cells have unique metabolic requirements as previously stated. Further, we are only now beginning to understand how the tumor's metabolism can impact T cell metabolism beyond nutrient deprivation and competition for glucose. The highly hypoxic tumor microenvironment promotes HIF1α expression in TILs, which further promotes glycolysis and decreased reliance on OXPHOS by the T cells. In general, TILs demonstrate major alterations in metabolism including defects in mitochondrial biogenesis and oxidative function (237, 241). Work from Delgoffe demonstrates how the state of the tumor (e.g., oxidative metabolism) can influence T cell responses to checkpoint blockade therapy and provide a predictive indicator to anti-PD-1 therapy responsiveness (241). Although tumor heterogeneity is often discussed in the context of antigen availability and "hot vs. cold" in terms of the presence of TILs, we may be overlooking the metabolic complexity of different tumor microenvironments and this significant contribution to T cell responsiveness. Better understanding the metabolic requirements of a highly effective TIL response in cancer and concurrently how the tumor metabolic requirements can be altered to generate a favorable TIL response could lead to an important convergence of anti-cancer therapies with a two-pronged approach.

Taken together, the studies summarized in this review highlight the complexities that must be considered when discussing and evaluating alterations in T cell responses and particularly when comparing memory formation with acute infection to conditions of chronic antigen stimulation. We are rapidly gaining greater insight into the molecular regulators of T cell dysfunction, effector generation, and memory development at both the transcriptional and epigenetic levels. Addressing how T cells interact with their microenvironment and the role of subsequent metabolic changes in the context of these important findings will be key in unlocking new strategies aimed at improving patient responses to chronic infections as well as cancer.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

ACKNOWLEDGMENTS

Figure 1 was generated in part by modifying and adapting individual Servier Medical Art (https://smart.servier.com/) in accordance with the Servier Medical Art by Service Creative Commons Attribution 3.0 Unported License.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 06 August 2019
doi: 10.3389/fimmu.2019.01717

Age-Based Dynamics of a Stable Circulating Cd8 T Cell Repertoire Component

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OPEN ACCESS

Edited by:
Alejandra Pera, Universidad de Córdoba, Spain

Reviewed by:
Jorg Goronzy, Stanford University, United States
Katherine J. L. Jackson, Garvan Institute of Medical Research, Australia
Spyros Kalams, Vanderbilt University Medical Center, United States

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Specialty section:
This article was submitted to Immunological Memory, a section of the journal Frontiers in Immunology

Received: *08 April 2019*
Accepted: *09 July 2019*
Published: *06 August 2019*

Citation:
Naumova EN, Yassai MB, Demos W, Reed E, Unruh M, Haribhai D, Williams CB, Naumov YN and Gorski J (2019) Age-Based Dynamics of a Stable Circulating Cd8 T Cell Repertoire Component. Front. Immunol. 10:1717. doi: 10.3389/fimmu.2019.01717

T-cell memory to pathogens can be envisioned as a receptor-based imprint of the pathogenic environment on the naïve repertoire of clonotypes. Recurrent exposures to a pathogen inform and reinforce memory, leading to a mature state. The complexity and temporal stability of this system in man is only beginning to be adequately described. We have been using a rank-frequency approach for quantitative analysis of CD8 T cell repertoires. Rank acts as a proxy for previous expansion, and rank-frequency, the number of clonotypes at a particular rank, as a proxy for abundance, with the relation of the two estimating the diversity of the system. Previous analyses of circulating antigen-experienced cytotoxic CD8 T-cell repertoires from adults have shown a complex two-component clonotype distribution. Here we show this is also the case for circulating CD8 T cells expressing the BV19 receptor chain from five adult subjects. When the repertoire characteristic of clonotype stability is added to the analysis, an inverse correlation between clonotype rank frequency and stability is observed. Clonotypes making up the second distributional component are stable; indicating that the circulation can be a depot of selected clonotypes. Temporal repertoire dynamics was further examined for influenza-specific T cells from children, middle-aged, and older adults. Taken together, these analyses describe a dynamic process of system development and aging, with increasing distributional complexity, leading to a stable circulating component, followed by loss of both complexity and stability.

Keywords: human CD8 T cells, computational immunology, repertoire maturation, circulation as depot, senescence

INTRODUCTION

Adaptive immune memory to pathogens arises by selecting particular lymphocyte clones from a pre-existing repertoire of naïve cells whose clonal antigen receptor has sufficient avidity to recognize the pathogen and initiate a response. Naïve lymphocytes show a high degree of species richness owing to the somatic rearrangement undergone by their antigen-specific receptor genes during their thymic development. Limited expansion of the thymocytes results in a relatively uniform naïve T cell frequency distribution, hence overall low diversity. Naïve cells are selected on pathogen in the periphery, and a portion of the expanded antigen-experienced cells are retained to guard against exposure to the same or similar pathogens. The original recruitment of adaptive

immunity in a response to pathogen is based on antigen presentation by innate immune cells that are primary responders and which play a predominant role in clearing the first exposure.

Upon re-exposure by the same or similar pathogen, immature adaptive memory will still be augmented by an innate inflammatory response, which will constitute the signal for continuing maturation of the adaptive system. Mature functional adaptive memory can be defined as the point when the innate cells are primarily required the antigen presentation and not the inflammatory response. This maturation process may require multiple exposures or immunizations against some pathogens. An early elegant description of the maturation process for B cells came from the work of Berek and co-authors, who showed the appearance of new, mutated IgH genes encoding high affinity antibodies only after multiple immunizations to the same antigen (1, 2).

T cell responses do not undergo the mutational maturation as do B cell responses and the primary evidence for maturation is the expansion in the number of cells involved in the response. Antigen experienced T cells are characterized by differential expression of activation and homing molecules (3). Further studies have established concepts such as effector vs. central memory (4, 5) and importantly the movement of memory T cells into depots and tissues (6–8). Analyses of immune and tissue depots have been recently described in man (9, 10). Memory maturation in humans has the added dimension of thymic involution (11) at puberty, which limits the number of new clonotypes that can enter the adult memory pool (12).

Our studies of the CD8 memory T cell repertoire take advantage of cellular expansion. The precursor frequency of the cells in the circulation is increased after expansion and the cells continue to show a strong replicative/survival response in culture when stimulated by a pathogen-derived peptide epitope. The latter can be considered as an additional *in vitro* exposure. Our focus has been on the CD8 T cell response to the conserved, matrix-derived, influenza epitope, $M1_{58-66}$. In individuals positive for human leukocyte antigen A2 (HLA-A2), this peptide drives a complex recall response. The distribution of the cells can be described as composed of two components when analyzed by rank frequency analysis (13). The first component is power law-like and the second component is composed of higher-ranking clones with typically only one exemplar per rank. The repertoire is characterized by use of the TRBV19 gene (hereafter referred to as BV19) which encodes Arg and Ser as part of the non-germ line component of the third complementarity determining region (CDR3) of the receptor (14–16). The CDR3 length is 11 amino acids and the RS appears at position 5. We have shown that the same complex clonotype distribution holds whether the cultures are further subdivided by their cytotoxicity, cytokine secretion, or binding of major histocompatibility complex (MHC)-bound antigen multimers (17). We have also shown that the distribution of the two components changes between middle-aged and older subjects (18). Recently, we have shown that the entire circulating CD8 BV19 repertoire, which subsumes the flu-specific repertoire, shows the same two component rank-frequency distribution as observed in the recall repertoire (19).

The first, power law-like, component of the distribution reflects the action of a repeated birth-death selection process (20, 21). It can also be viewed as affinity-based selection for replication of a set of cells that are initially normally distributed with respect to affinity for a ligand (13). The second component of the MI_{58-66}-specific and BV19-specific repertoires has posed a puzzle as to its significance. It could represent a secondary selective expansion process. However, a simpler explanation is that the second component reflects a differential abundance of well-selected clonotypes in the circulation. We therefore expect that such clonotypes, in addition to being sampled at higher than expected frequencies, will be stable over time. We also expect that the second component will be a function of age as it is unlikely a stable repertoire component can precede the establishment of the initial complex repertoire.

Here we use a measure of clonotype stability of circulating BV19 clonotypes from five adult subjects to show that the second distributional component is indeed stable. We then go in to show the same relation can be observed for recall repertoires. Furthermore, the circulating stable component is not observed in children, and is present in a degraded form in older adults. The results are discussed in terms of repertoire development and senescence. The significance of a circulating pool of CD8 T cells is also discussed.

MATERIALS AND METHODS

Study Cohorts

Peripheral blood mononuclear cells (PBMC) were collected from five healthy child subjects (C1, C2, C3, C4, and C5), six healthy middle-aged adult subjects (mA1, mA2, mA3, mA4, mA5, and mA6) and six older adult subjects (oA1, oA2, oA3, oA4, oA5, and oA6). All subjects were typed as HLA-A2.1-positive. Ages at time of enrollment, number of blood samples and average time span between samples for the *ex vivo* sequencing and *in vitro* recall studies are provided in **Table 1**. The timing of sample collection relative to the date of first sampling is provided in **Supplemental Table 1** and illustrates the spacing between individual measurements and general overlap across study subjects. This timing data shows that our estimates of stability are derived under similar conditions for each person. Because we are interested in steady state conditions, samples were used from time periods during which the subjects did not report flu-like illness since the previous sampling. A subset of adult subjects performed bi-weekly self-administered swabs during the local the flu season. The samples used here were not taken from samples collected after a swab positive for influenza.

The healthy child subjects were enrolled under protocol Children's Hospital of Wisconsin IRBnet: 116305 "Generation and decay of memory T cells in children with Juvenile Rheumatoid Arthritis and healthy siblings following administration of trivalent inactivated influenza vaccine," from the Children Hospital of Wisconsin. The subjects analyzed here were the controls in this study. The adult subjects were enrolled under protocols authorized by the Institutional Review Board of BloodCenter of Wisconsin: BC 05-11, "Generation and Decay of Memory T Cells in Older Populations," and BC 04-22,

TABLE 1 | Age and sample collection data of the study cohorts.

		Subject ID	Age at first blood sample (in years)	Number of blood samples	Average time span between samples (in months)
Ex vivo HTS	Adults	oA1	68	6	3.77
		mA1	39	7	2.94
		mA2	40	6	3.31
		mA3	40	5	2.55
		mA4	44	6	3.37
		Average[§]	46.20 ± 12.34	6.00 ± 0.71	3.19 ± 0.46
Recall	Children	C1	7	8	3.79
		C2	9	5	7.98
		C3	10	6	3.77
		C4	12	8	3.79
		C5	14	7	3.79
		Average	10.40 ± 2.70	6.80 ± 1.30	4.62 ± 1.88
	Middle-aged adults	mA1	39	10	2.65
		mA2	40	8	3.44
		mA5	40	8	2.49
		mA6	48	10	4.43
		Average	41.75 ± 4.19	9.00 ± 1.15	3.25 ± 0.89
	Older adults	oA1	68	8	1.94
		oA2	78	8	5.69
		oA3	69	9	3.56
		oA4	78	13	3.81
		oA5	80	5	5.36
		oA6	78	8	4.56
		Average	75.13 ± 5.23	8.50 ± 2.59	4.15 ± 1.37

[§]*Indicates mean ± standard deviation.*

"Robust T Cell Immunity to Influenza in Human Populations." These protocols have been transferred to the IRB of the Medical College of Wisconsin (MCW). Written informed consent was obtained from participants, or their parents/legal guardians in the case of children.

$M1_{58-66}$ Recall Culture and Clonotyping

PBMC were isolated using Ficoll-Paque plus (Amersham Biosciences) and stored frozen under liquid N_2 until used. The $M1_{58-66}$ peptide (GILGFVFTL) from the M1 protein of influenza A virus was synthesized by The Blood Research Institute Peptide Core. The procedure for the culturing PBMC, CD8 cell selection, nucleic acid preparation, amplification, cloning, and sequencing has been described previously (17). The recall analyses were performed as part of our general human immunology studies. PBMC were stimulated at 1×10^6 cell/ml in 2-ml cultures with $M1_{58-66}$ peptide added to 1 μM final concentration in complete RPMI media supplemented 10 U/ml of recombinant IL2 and 10% human pooled AB sera in round bottom tubes or wells for 7 days. On day 3, an IL2 supplement (10 U/ml) was provided. On day 7 non-adherent cells were collects after agitation, counted and re-plated with an equivalent number of fresh irradiated autologous PBMC at 10^6 cells/ml. The feeders had been prepulsed with peptide (1 μM final concentration). IL2 was added to 10 U/ml. Another 7-day culture was performed with IL2 addition at day 3. However, the analysis for subject mA6, and for most of the child samples was performed with our dendritic cells (DC) protocol in which adherent cells are prepared by overnight culture. Half of these adherent cells (monocyte derived APC) are used for the first week stimulation of the non-adherent PBMC (i.e., lymphocytes), and the other half maintained in IL4 and GM-CSF for use in the second week. All cultures in adults were in triplicate, and predominantly in duplicate for the child cohort owing to smaller blood sample volumes.

After two 7-day cycles of recall culture, CD8 cells are isolated by magnetic beading using Dynal CD8 positive isolation kit (Invitrogen Inc., Carlsbad, CA) according to manufacturer's instruction. mRNA samples were isolated from the CD8 cells using Dynal Oligo (dT) beads according to manufacturer's instructions (Invitrogen). cDNAs was prepared using a poly-T primer and MMLV reverse transcriptase (Invitrogen). All cDNAs were titrated using a pair of C-region primers, one labeled with fluorescein, in three PCR reactions for 20 cycles, each reaction using a doubling of the cDNA concentration. The cDNA for BV19 analysis was used at the concentration corresponding to the midpoint in the linear plot of cDNA concentration to amplicon fluorescence intensity using cDNA concentrations where the amplicon fluorescent intensity increased in direct relation to the cDNA concentration. The PCR used our standard BV19 and BC primers (22). The BV19 primer concentration was 20 times the concentration of the C-region primers used in the titration to ensure the same efficiency. As long as the experiments are performed under these conditions, they should provide representative data about the sample. Since all samples obtained from humans are far from exhaustive, representative data is all that can be expected.

We chose CD8 selection after having examined the outcomes of separating the cells based on CD107 expression as a marker for degranulation/cytotoxicity function and $M1_{58-66}$:HLA-A2 multimers as a TCR affinity marker. We observed that each of these showed a complex repertoire, but they were not completely overlapping. Hence CD8 represented the broadest selection and was the simplest to use as well (17), which is important when large number of samples are involved.

The PCR product was cloned into *E. coli* using pCR4-TOPO Cloning Kit (Invitrogen, Carlsbad, CA). Bacterial colonies (∼400) were grown overnight and sent to Agencourt Bioscience (Beverly, MA) for sequencing. Sequences were received in *fasta* format and analyzed using "CDR3Reader" software, which identified V and J regions, assigns clonotype names according to our convention (23), and counts occurrences of each clonotype. The identity of a distinct instance of a β-chain is based on the rearrangement site with respect to each of the two rearrangements that generated the chain, D to J and V to DJ. The region between the sites is referred to as the NDN region which represents the junctional diversity present in all the β-chain genes that underwent the same D to J and V to DJ choice. The NDN region is embedded in the CDR3 (24), which is composed of all the amino acids between the conserved cysteine at the c-terminus of the V gene and the conserved phenylalanine-glycine in the J

region. The naming convention provides the information as to which V and J regions were used, the sequence and encoding of the NDN as well as the length of the CDR3.

Data analyzed represents pooling of the duplicate and triplicate cultures. Although the colony counting procedure involves ligation and bacterial transformation steps, the results are reproducible as tested in experiments in which large cultures were divided in three and each portion subject to CD8 selection, bacterial cloning and sequencing. There was an excellent clonotype overlap between the three separate assays of the same culture [Supplemental Figure 1 in (17)].

It should be pointed out that our definition of clonotype is only based on the TCR β-chain. Most T cells only express one β-chain, referred to as allelic exclusion, so this is a close one to one mapping. However, after thymic β-selection, the DN thymocytes expand prior to α-chain gene rearrangement (25, 26). Thus, cells with the same β-chain may have different α-chain partners, with cells with each distinct β-α pair representing a separate clonotypic lineage. Thus, our description of diversity is an underestimate, as our analysis would group all of these as one lineage.

High Throughput Sequencing (HTS) of BV19 TCR

T cell sequence analysis is described in more detail elsewhere (19, 27), including error estimation, and steps taken in cleaning the nucleotide sequence data, defining motifs and motif distributions. In brief, PBMC from five to seven different time points per subject were used (**Table 1**, *ex vivo* HTS panel). PBMC were thawed, CD8 cells collected by magnetic bead separation and mRNA and cDNA prepared as described above. PCR amplification was done using our standard BV19 and BC primers modified to include the Roche 454 adapter sequences and sample ID tag sequences. Owing to the higher concentrations needed for 454 sequencing in lieu of scaling up, multiple amplifications were performed, each equivalent to the reactions used for the cultures. The concentration of purified PCR products was measured using NanoDrop-1000 spectrophotometer. From 6 to 12 purified PCR products were pooled to obtain a total of 2,500 ng. The samples were further amplified and prepared for high throughput sequencing at the Human and Molecular Genomic Center (HMGC) Sequencing Facility (www.hmgc.mcw.edu) of Medical College of Wisconsin. The sequencing was performed on the Roche GS-FLX Genome Sequencer using Titanium chemistry. Samples were coded by identifier sequences embedded in the primers. After decoding, sequences derived from each sample were downloaded in *fasta* format and analyzed using "CDR3Reader."

The HTS data differs from the recall data in the presence of two power law-like components in the rank-frequency analyses. The method used did not include a unique molecular identifier as part of the cDNA or second strand synthesis (28). With the additional amplification associated with Roche 454 sequencing it is very likely that the shift to higher ranks of the second component is associated with the concentration of cDNA (sample) being analyzed and the number of amplification cycles. Decreasing the concentration of cDNA under identical experimental conditions enhances the shift (unpublished), thus our analyses were restricted to using sufficiently high concentrations of cDNA that minimized this effect. This implies starting with a sample size sufficient to clearly observe the lower ranks.

Data Analysis

The repertoire data from any sample can be tabulated as the clonotype name and the number of observations of that clonotype. Data from such a table can be used to define some key repertoire measures: number of clonotypes, N, number of observations, M, number of clonotypes observed once (i.e., singletons), N_S, the highest ranking clonotype, $Rmax$. Rank frequency analysis involves counting the number of clonotypes observed once, twice, thrice, to $Rmax$. These measures in turn can be used to generate a number of repertoire characteristics. We use: (1) N as a general proxy for richness, (2) $\frac{M}{N}$, observations per clonotype as a proxy for abundance, (3) $\frac{Ns}{N}$, the fraction of clonotypes observed once (i.e., rank $= 1$) to describe the singleton tail of the distribution, and (4) $\frac{Rmax}{M}$, the proportion of observations due to the highest ranking (i.e., most frequently observed) clonotype. These four characteristics offer a general overview of the distribution as they provide an average richness and abundance and a description of the two extremes.

We used a similar approach to clonotype temporal stability within the repertoire. The stability of the clonotype is defined as the number of times it was observed across repeated measurements. Clonotypes observed at all times analyzed are considered stable and clonotypes observed only at one time unstable. Because the measurement of temporal stability is based on multiple time points, more time points should provide a better estimation of stability. To compensate for small differences in the number of time points, we introduce a relative stability characteristic of the repertoire in which observation at one time is equal to a stability of 0, observation at all times is equal to a relative stability of 1. Thus, the relative stability of a clonotype is calculated as: $\frac{number\ of\ times\ observed - 1}{maximum\ possible\ number\ of\ times\ observed - 1}$. The average relative stability of all the clonotypes at a given rank is the average of all the individual relative stabilities of clonotypes at that rank. Thus, clonotypes with rank lower than six in the pooled repertoire that consists of six time points cannot be stable. To assess the relationship between clonotype distributional frequency and the estimates of temporal stability we used correlation analysis and provided correlation coefficients, R and coefficients of determination, R^2.

While our data sets are of similar size, the number of observations (M), and clonotypes (N) can vary, we used a number of normalization procedures. Normalized rank can be used for plotting the relation to normalized rank frequency or to average relative stability. We normalize each *ln*-rank by dividing the log-transformed values of $Rmax$; the latter representing the largest rank possible. The extreme values in this case are 0 for $\frac{\ln 1}{\ln Rmax}$ and 1 for $\frac{\ln Rmax}{\ln Rmax}$. For rank frequency, we normalize the *ln*-rank frequency by dividing by the highest frequency component which is when the rank $= 1$ (singleton clonotypes $= Ns$). This spreads the data from one to zero, with one reflecting

the contribution of the highly abundant singleton clonotypes, resulting from $\frac{\ln Ns}{\ln Ns} = 1$, and the frequency of the highest ranking clonotype (usually one) equal to zero; $\frac{\ln 1}{\ln Ns} = \frac{0}{\ln Ns} = 0$. This normalization procedure works best for comparison of power law-like distributions. The normalized rank and rank frequency relationships were formally tested using an anchored power-law regression model, in which we regressed normalized *ln*-rank frequency y against normalized *ln*-rank x as follows: $y = 1 - (1 - (1 - x)^u)^v$, where u and v are the power-law parameters that govern the relationship curvature.

The data collected here represent clonotype numbers and frequencies that were a function of the cDNA input used to generate the amplicons used for the subsequent Roche GS-FLX Genome Sequencer analysis. Increasing or decreasing the concentration of cDNA increase or decreases the number of clonotypes identified and the frequency of the low ranking clonotypes as well as the maximum rank. Data were analyzed using Microsoft Excel and RStudio. Our definition of "clonotype" as used here has been qualified above.

The clonotype datasets generated and analyzed here are available as **Supplemental Data 1** as is the approach for deriving the CDR3 nucleotide sequence from the clonotype names (**Supplemental Figure 1**).

RESULTS

Role of Clonotype Rank as a Proxy for Selection

A repertoire is composed of clonotypes, which are defined by the clonal rearrangement of the receptor genes. As the clonotype is peripherally selected it expands and thus has an increased frequency within the repertoire. The frequency is measured by the number of observations after a controlled amplification of the receptor genes or transcripts. As long as the frequency is attributed to the entity "clonotype," the analysis of the repertoire is limited to counting the clonotypes and/or some characteristic thereof. A higher level of analysis is obtained if the repertoire is described by a frequency of frequencies. In this approach, the absolute or relative measurement of the clonotype defines the rank of the clonotype. Thus, the repertoire (a pathogen-specific ecosystem) can be viewed as a collection of clonotypes (species) whose previous successful selection defines their rank (abundance). We will be sampling this system indirectly, from the circulation, and our samples will represent a small portion of the overall repertoire. Therefore, our quantitation will be relative but should reflect proper relationships as long as we do not skew the counting process by the methodology used to amplify the signal. The methods section describes the precautions we take to be in the proper relation of starting cDNA and amplification cycles.

As we have previously described (13, 17, 18, 27), the rank-based description of the repertoire shows that the highest frequency of responding clonotypes is that of clonotypes representing the lowest rank, i.e., those measured once. A log-log transformation of the rank vs. rank-frequency data shows a two-component plot with one component decreasing in a linear manner, and the second consisting of a number of mostly single clonotypes at very high ranks. The first component is indicative of a power law-like distribution fitting the equation $y = ax^b$. The exponent, b, describes the distribution of the rank frequency of the clonotypes in the repertoire as it descends from the lowest to highest ranks. In the log transformation of the above equation, $\log y = b^* log\ x + a$, parameter b represents the slope of the data which is approximately linear. Parameter a represents the proportion of clonotypes that constitute the lowest rank, and is the y intercept of the line.

The thymus produces an initial repertoire that is relatively uniform, with skewing due to increased probability of certain aspects of the rearrangement mechanism (29) along with the limited expansion after β-selection (26, 30). Our initial description of how a power law-like distribution may arise from an initially uniform distribution was very focused on the TCR but actually represents a general phenomenon. With a random uniform distribution of receptors, a measure of affinity for a particular ligand will be normally distributed (31). A left-censored normally distributed distribution represents positive affinity with the maximum number of receptors being neutral and a small number representing the high affinity. An example for ∼4,000 clonotypes representing a left-censored distribution is shown in **Figure 1A** (filled circles). All that needs to be done is to postulate that affinity will correlate with response, which includes cell division (32, 33). This can be thought of as a reward function for the lymphocyte network. A reward function resulting in 12 divisions (3–4 days) for the highest affinity (2^{12} ∼ 8,000 cells) and no divisions, but survival, for neutral affinity, is shown (**Figure 1A**, open circles). The resultant repertoire distribution (**Figure 1B**) shows a power law-like distribution. Hence, an initial uniform distribution of clonotypes that display a normal distribution with respect to affinity to ligand can give rise to distribution with power law-like characteristics on the basis of selective cell division, with the clonotype rank describing the selection.

An important characteristic of a power law-like distribution is that it is scale free. From a sampling perspective, this means that doubling the amount of cells, will generate the same distribution pattern with some of the cells that were observed once now being observed twice, some that were observed twice now three times, etc. If we only use half the cells, we lose some singletons, some doubletons become singletons, etc. However, as long as the PCR cycle number is decreased, the distribution remains the same and is still representative. Without compensating the cycle number, the data becomes skewed owing to over-amplification.

However, the immune response is also characterized by a reduction of the expanded population after pathogen clearance, which we have modeled as a birth-death process (20, 21). The birth-death model more closely approximates our actual observations. Of course, even the birth-death model does not incorporate other factors like signaling thresholds, nor does it address possible probabilistic approaches to cell division which would require counting numbers of APC-T cell interaction, or numbers of exposures. However, it is clearly a guiding principle for arriving at a power law-like distribution from a uniform normal distribution and shows the usefulness of approaching repertoires using clonotype rank as a descriptor.

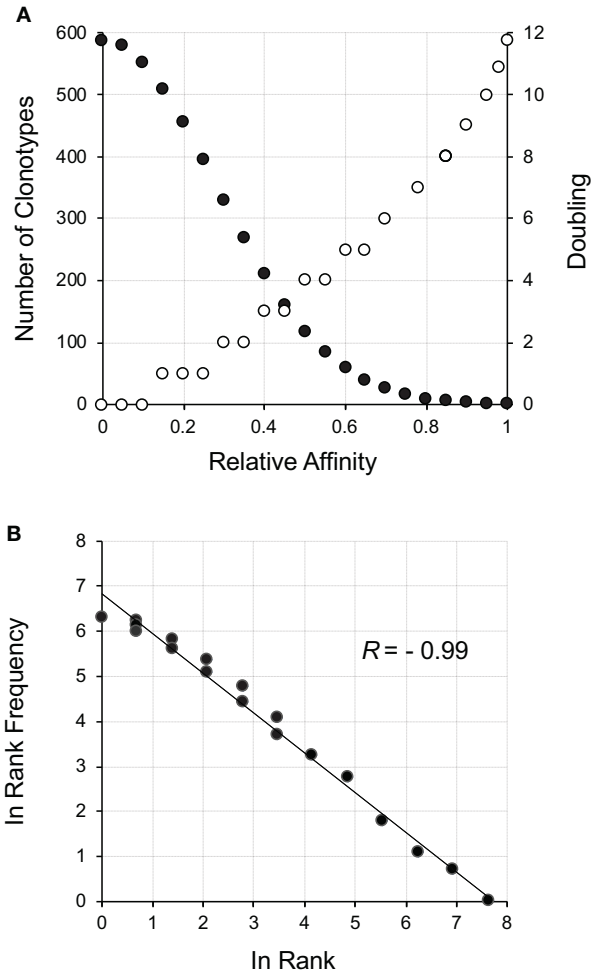

FIGURE 1 | Power law-like distribution as a result of a reward function applied to a starting clonotype population normally distributed with respect to affinity. **(A)** The neutral to positive affinity portion of a normally distributed ($\sigma = 0.14$) population of clonotypes is shown as filled circles. The corresponding reward function resulting in the number of cell divisions is shown as empty circles. Since the division process is discrete, a range of affinities can fall within a particular doubling threshold. The reward function was set for a maximum of 12 divisions in equally distributed steps across the affinity spectrum. **(B)** The reward was applied to the number of cells at each affinity increment and the rank frequency of the resulting distribution calculated and plotted.

Rank-Frequency Distribution of Adult BV19 Utilizing CD8 T Cells

Our initial analysis of complex repertoires utilized the recall response to influenza $M1_{58-66}$. Circulating CD8 T cells expressing the BV19 TCR represent the next higher level of repertoire structure that encompasses this response. Analyzing the BV19 repertoire would represent a generalization of our findings. Indeed, an initial HTS analysis of BV19 CD8 TCR from an adult subject showed a similar complex clonotypic distribution to our previous recall data (19). Here we have added the HTS analysis of pooled circulating BV19 CD8 T cell repertoires from four middle-aged adult subjects, mA1 to mA4. The subject age and average sampling data are given in **Table 1** (*ex vivo* HTS panel). Sample collection relative to the timing of the first sampling is provided in **Supplemental Table 1**. In all cases the pooling was of samples collected on average every 2 months, and the period of elapsed time between first and last samples is approximately a year and a half. Standard repertoire measures and characteristics for the pooled repertoires, described in the Method section, are provided in the top panel of **Supplemental Table 2**. The data from the five subjects differed slightly in depth of analysis at the level of number of observations, *M*. We also provide a summary of the repertoire measures and characteristics for the individual samples in the form of average values and deviations in the bottom panel of **Supplemental Table 2**. As might be expected, there was some variability in the measures obtained at the different time points for all subjects but overall the values are comparable.

The rank frequency analysis for the pooled repertoire data for all five subjects is shown in **Figure 2A**. The rank frequency plots are very similar, and each has a power law like component and a second high-ranking component which are demarcated at rank *ln* 5 by a vertical line. Thus, *ln* 5 corresponds to the critical point dividing the power law-like component(s) from the high-ranking component. As noted previously (19), the power law-like component appears to have two parts which are divided at $\sim$ *ln* 2. It should be pointed out that the data were generated without using unique molecular identifiers (28) and it is very likely that the two parts of the power law-like component observed in the rank-frequency analysis is a function of sample concentration to amplification cycle ratios. All five BV19 repertoires show a similar clonotype distributional frequency profile with the average correlation coefficient of -0.83 ($R = -0.83 \pm 0.03$ and $R^2 = 0.68 \pm 0.05$). Individual R and R^2 values are shown in **Table 2**, in the section labeled "Rank vs. Rank Frequency **Figure 2A**."

Clonotype Stability

Clonotypes in a pooled repertoire have a measure describing the number of times they are present among the different sample times, which can define the stability of the clonotypes in the repertoire. This, measure is defined by the number of time points (increments) at which the clonotype was observed. With a pooled repertoire representing a number of distinct sampling times, a clonotype that is observed once is considered unstable and one observed at all times is considered stable. The temporal stability of the pooled repertoire is defined by the number or relative frequency of the clonotypes at each stability increment. The repertoire temporal stability data is shown for all five subjects in **Figure 2B**. The number of possible observation times (stability) is on the x-axis. The number of clonotypes at each stability increment is plotted on the y-axis using a logarithmic scale and the percentage of clonotypes observed only once is shown above the first bar. Most clonotypes are only observed once, indicating their temporal instability.

The repertoire stability is characterized by a decreasing frequency of clonotypes at higher stability increments. This was examined in more detail by plotting the natural logarithms of stability and clonotype frequency at each stability increment (**Figure 2C**) which showed that this relation is also power

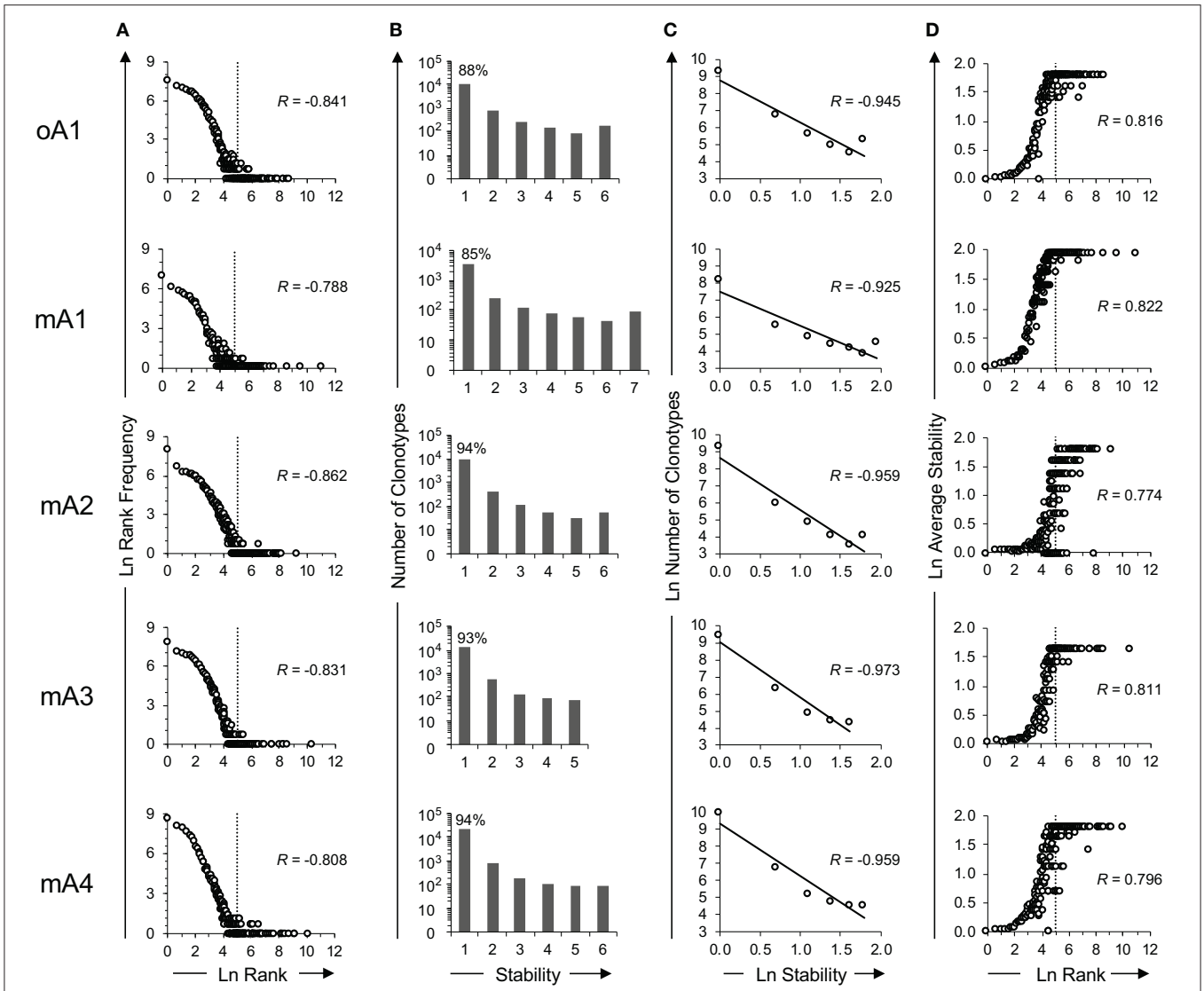

FIGURE 2 | Time series analysis of the *ex vivo* BV19 repertoires of five adult subjects using high throughput sequencing. **(A)** Natural log-transformed clonotype ranks vs. rank frequency. The inflection point in the graph is identified by vertical dotted lines at *ln*-rank 5. **(B)** Repertoire stability data. The absolute number of clonotypes is shown for each stability increment. The clonotype count (y-axis) is on a $\log_{10}$ scale. The percentage of clonotypes observed at one time is shown above the bar graph. **(C)** The natural log-transformation of the data in panel B. **(D)** The log-transformed average stability is plotted as a function of *ln*-rank. The vertical lines show the two rank components defined by their inflection points of the distributional curve. The *R* values, where shown, describe the coefficient of correlation.

law-like. The value of the negative correlation between *ln* stability and *ln* number of clonotypes ($R = -0.95 \pm 0.02$ and $R^2 = 0.91 \pm 0.03$) is very similar for all the subjects, irrespective of the variation in the numbers of times sampled or number of observations and clonotypes between the individuals. This similarity implies that we are defining a fundamental characteristic of the repertoire. The *R* and R^2 values associated with **Figure 2C** and the means and standard deviation for these data sets are given in **Table 2**: Stability vs. Number of Clonotypes.

It was of interest to examine the relation of the stability measure with relation to clonotype rank. This is done by calculating the average stability for all the clonotypes observed at a particular rank. **Figure 2D** shows that stability increases as the rank increases. The *R* values are shown for each subject and the average of $R = 0.80 \pm 0.02$. The R^2 values are provided in **Table 2** section "**Figure 2D**" and the average of $R^2 = 0.65 \pm 0.03$. For all five subjects, there is a rank after which the clonotypes are all stable although the extent of this fraction can vary from subject to subject.

Comparison of Clonotype Rank Frequency and Stability as a Function of Rank

Examining **Figures 2A,D** indicates that there may be a direct relation between rank-frequency and rank-stability. This relation

TABLE 2 | Coefficients of correlation (R) and determination (R^2) between: (1) rank and rank frequency, (2) stability and number of clonotypes, and (3) rank and average stability for each individual within the study cohorts in reference to **Figure 2**.

	Rank vs. rank frequency		Stability vs. number of clonotypes		Rank vs. average stability	
	R	R^2	R	R^2	R	R^2
Subject ID	**Figure 2A**		**Figure 2C**		**Figure 2D**	
oA1	−0.841	0.708	−0.945	0.893	0.816	0.665
mA1	−0.788	0.621	−0.925	0.855	0.822	0.676
mA2	−0.862	0.744	−0.959	0.921	0.774	0.599
mA3	−0.831	0.691	−0.973	0.946	0.811	0.658
mA4	−0.808	0.653	−0.959	0.92	0.796	0.633
Average[§]	−0.83 ± 0.03	0.68 ± −0.05	−0.95 ± 0.02	0.91 ± 0.03	0.80 ± 0.02	0.65 ± 0.03

[§]*Indicates mean ± standard deviation.*

was analyzed by generating a measure of normalized rank and plotting either normalized rank frequency (**Figure 3A**) or normalized average stability (**Figure 3B**) as a function of normalized rank. The normalized rank frequency plots for each subject differed slightly with respect to slope and inflection point between the second and third component. The stability plots showed the same relative differences resulting in a striking symmetry between the two data sets. Stability is a function of increasing rank which is inversely associated with frequency of clonotypes at that rank. There is some noise, defined as a spread of a particular stability level over a number of ranks, in the stability data. The significance of the noise, which is most apparent in the data from subject mA2, is still not clear. Overall, the data show that clonotype stability together with clonotype distribution are integral properties of overall repertoire complexity.

The stability measures and characteristics of the five pooled repertoires are provided in **Supplemental Table 2** and **Figure 2B**. While the fraction of stable clonotypes varies between 0.004 and 0.021 (average ∼0.01) this small fraction of stable clonotypes can represent an average of ∼0.38 of the observations (proportion of stable clonotypes, $\frac{Mst}{M}$). Thus, a large part of the circulating BV19 CD8 T cells are composed of a small number of very stable clonotypes.

The results of these *ex vivo* analyses of adult CD8 repertoires show that there is a small percentage of clonotypes representing a large percentage of T cells that are represented in the circulation at all times, which is compatible with a stable circulating depot of cells. This also would explain the different distribution of the second rank-frequency component. For this component, rank is not just a function of previous expansion but also of accessibility.

Stability of the $M1_{58-66}$ Recall Repertoire in Subjects Representing Three Age Cohorts

The BV19 data reflects a comprehensive description of a large number of clonotypes of unknown specificity at a particularly point in development. We hypothesized that the complexity observed in the adults is part of a dynamic process of evolution and devolution over a lifetime (21). To examine this hypothesis, we focused on the memory component as defined by recall of the $M1_{58-66}$-specific CD8 BV19 T cell repertoire from samples obtained from child, middle-aged, and older adult cohorts (**Table 1**). The repertoire consists of the canonical clonotypes whose receptor encodes Arg and Ser in the correct location of the non-germline-encoded portion of the receptor. Because we are focusing on stability as a steady-state phenomenon, time points were chosen for the recall analysis to avoid including samples after a suspected or proven influenza exposure. Immunization with trivalent flu vaccine does not appear to have an effect on the $M1_{58-66}$ repertoire, which is not unexpected as it is not part of the vaccine.

We have previously analyzed single time points from two adult cohorts representing middle-aged and older individuals (18). These both show similar two component rank-frequency data that differ in the proportion of singleton clonotypes, lower for the older cohort, and position of critical point between components, left-shifted in older adults.

Here we present the analysis of the clonotype stability of some of the same individuals as well as others in the same age cohort and we have also provided data from a child cohort. The individual sampling data for the three age cohorts are provided in **Table 1**. The measures and characteristics of the recall repertories of the subjects in each of the three cohorts is provided in **Supplemental Table 3** for the child cohort, **Supplemental Table 4** for the middle-age, and **Supplemental Table 5** for the older adult cohorts. Average values of the measures and characteristics for each cohort is provided in **Supplementary Table 6**.

To help visualize the expected and actual outcomes of the stability analysis, the data are plotted as the natural *log* of stability, and the lower x-axis is annotated in terms of the stability increment, counting the number of sampling times in each analysis. The actual *ln* values are shown on the upper axis. A tick mark without an associated data marker represents a missing value. The BV19 RS L11 recall repertoire shows a decreased frequency of clonotypes as the stability increment increases. The regression analysis shows an excellent linear fit ($R = -0.98 \pm 0.01$ and $R^2 = 0.96 \pm 0.03$), and therefore the relationship

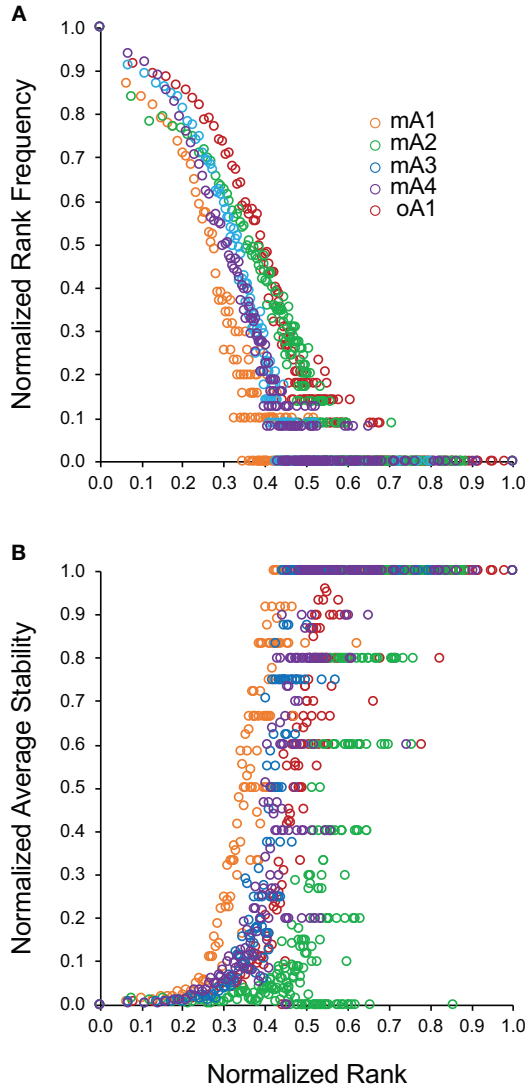

FIGURE 3 | Relationship between normalized clonotype rank and either **(A)** normalized rank frequency or **(B)** normalized average stability for five adult subjects. Subject data is identified by different marker colors as shown in the insert of **(A)**.

between stability and clonotype frequency can be described as power law-like (**Figure 4A** and Panel 1 in **Table 3**). Importantly, none of the five child subject repertoires had $M1_{58-66}$-specific clonotypes that were stable; i.e., observed at all times sampled. The missing values represent the highest stability increments and the number of missing values varies from subject to subject. These data indicate that the clonotypes involved in the response are starting to show signs of increasing stability but have not yet generated a completely stable clonotypic subset of the repertoire. Even though complete stability is not attained, the stability data is described as a power law-like distribution, as was observed for the BV19 *ex vivo* adult data.

Stability of the $M1_{58-66}$-specific clonotypes in middle aged-subjects (**Figure 4B**) was similar to the BV19 *ex vivo* repertoire data, of which these clonotypes are a subset. There is power law like distribution with an increase in frequency at higher stability increments. The regression analysis shows an excellent fit of the data and a high overall correlation ($R = -0.89 \pm 0.07$, Panel 2 in **Table 3**).

The stability data of older subjects is shown in **Figure 4C** and Panel 3 in **Table 3**. The data for oA2 is most similar to the middle-aged data. However, there are two-time point increments, 4 and 6, for which there are no values, indicating a loss of stability. Subject oA1 and oA3 can be considered to have a child-like pattern, in that there are no clonotypes present at the highest three or four stability increments. Subject oA4, oA5, and oA6 show an intermediate pattern in which there is a reversion to a child-like pattern, with the maintenance of some of the high stability pool of clonotypes. Thus, the older adult data indicates an interesting interrupted pattern in the clonotype stability pattern as would be expected from senescence of independent pools.

Comparison of Recall Clonotype Rank Frequency and Stability as a Function of Age

Rather than trying to compare 15 panels each of rank frequency and rank stability data analyzed as five- (child), four- (middle-aged), and six- (older adult) clusters, we generated a cohort specific summary for rank vs. rank frequency (**Figure 5A**) and for rank vs. stability (**Figure 5B**). The recall repertoire data was generated by binning the normalized rank values of all subjects in a cohort in increments of 0.05 and averaging the individual rank values as well as the corresponding frequency or stability values associated with each bin. The data were fitted as anchored regressions, defined by parameters u and v using the formula, $y = 1 - (1 - (1 - x)^u)^v$. The parameter u controls the concave aspect of the curve, whereas v reflects how evenly points are distributed on the curve. With both u and v at unity, the data would constitute a straight line between (1,0) and (0,1).

For the child cohort, the parameter pair of $u = 1.735$ and $v = 0.855$ is indicative of a slight concave deviation from a 45° linear slope (**Figure 5A**, red circles). For the adult cohort, the pair of $u = 3.3$ and $v = 1.25$ is characteristic of a steep curvature; and for older cohort the pair of $u = 5.8$ and $v = 0.9$ is characteristic for a steady slope for the low-frequency component and a very steep curvature for a high-ranking component (**Figure 5A**). The values for v for the child cohort resemble values for the older adult cohort indicating similar properties of the low-frequency component in the repertoire distribution. The v value is indicative of the regularity of the rank values along the curvature; with the child cohort showing a relatively even spread, whereas the older adult values are densest in the high-frequency portion of the curve. The two adult cohort datasets show very similar patterns of distributional complexity to those we described in a previous study (18) using samples only collected at one time.

The normalized average stability values for the three cohorts (**Figure 5B**) shows that for the middle-aged adult cohort, the high ranking clonotypes are consistently and strongly associated with

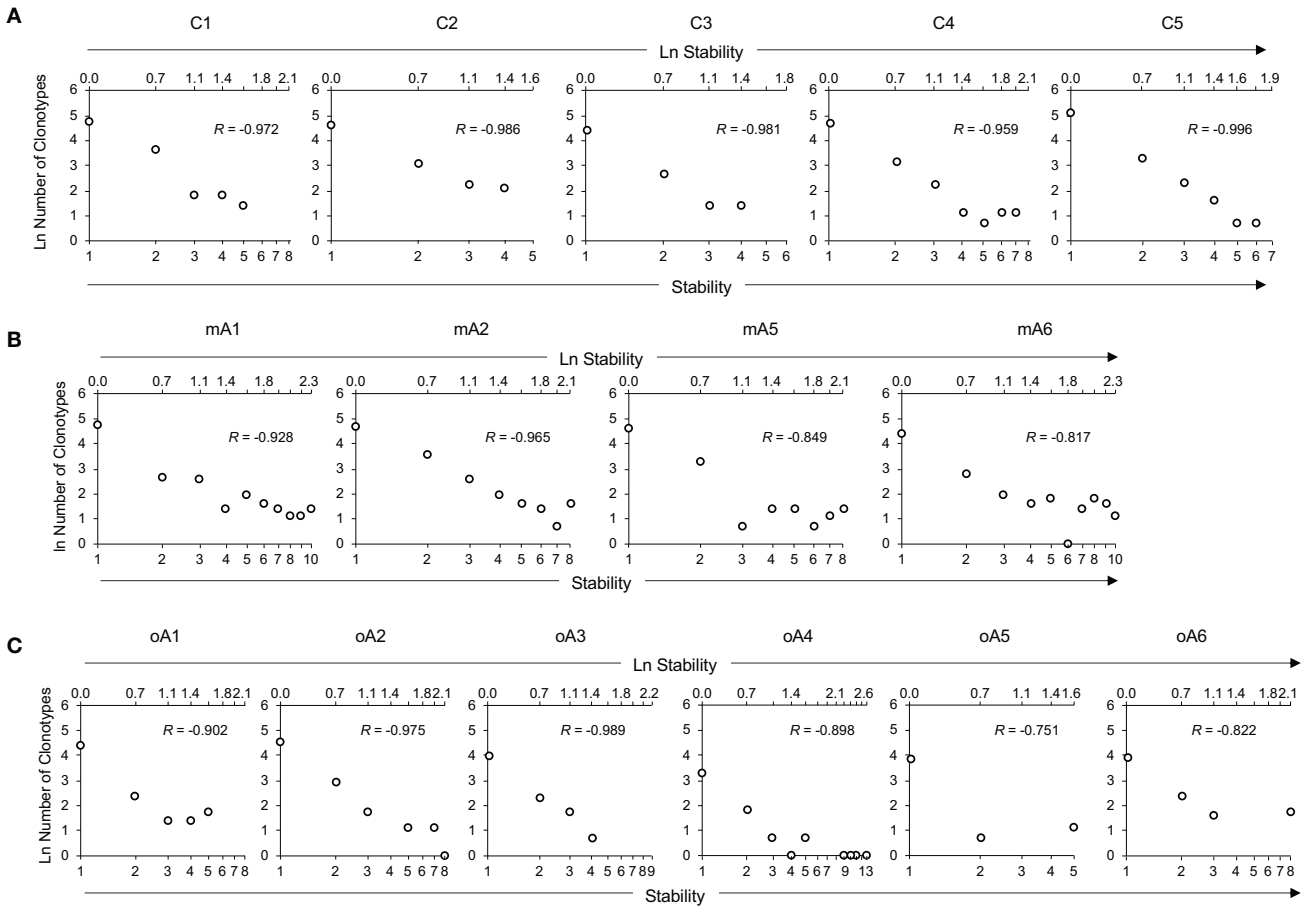

FIGURE 4 | Repertoire stability data from $M1_{58-66}$ specific recall analyses of **(A)** five child subjects, **(B)** four middle-aged adult subjects, and **(C)** of six older adult subjects. Repertoire stability data is shown as the natural log-transformed stability increment vs. number of clonotypes at that stability. The lower x-axis indicates absolute values of stability increments. The upper x-axis is demarcated in terms of the *ln* of the stability increments. Lack of a datapoint at a stability increment represents a missing value. Subject identifiers are shown above each set of panels. The *R* values represent coefficients of correlation for each dataset.

maximum stability ($R^2 = 0.91$). The child cohort is characterized by lower stability with none of the highest ranking clonotypes were observed at all times examined ($R^2 = 0.85$). The older adult cohort data (blue diamonds) shows a similar trendline to that of the child. However, the spread of stability values is quite wide at the high ranks ($R^2 = 0.71$). This heterogeneity is due to individual differences within the cohort with some subjects having stable clonotypes at higher ranks while others do not (**Figure 4**). This is linked to the higher density of values at higher ranks in this cohort noted above. In spite of this higher density of high ranking clonotypes overall stability is lost indicating that in this cohort the relation between stability and rank is broken.

The recall data provide a focused examination of antigen-specific repertoire characteristics, but also reflect the nature of the functional definition of specificity. The data are summation of the complex distribution in the PBMC as well as the *in vitro* survival and growth potential of these cells. The latter may vary based on how many previous divisions the cells had already undergone, or requirements for costimulation that are not being met in the culture conditions. While the specific nature of the recall measurements may come at the price of additional heterogeneity, the stability and complexity measures show a definite change of a peptide-specific repertoire with age.

DISCUSSION

The analysis of CD8 T cell repertoire evolution presented here defines a new measurable characteristic of the circulating repertoire, clonotype stability, and shows that stable clonotypes make up a sizable fraction of the mature circulating CD8 BV19 repertoire. The symmetrical relation between the stability and rank frequency provides an explanation for the previously noted division of clonotype rank frequency into at least two distributional components when examined by rank frequency analysis. We propose that the first distributional component representing a power law-like distribution in the circulation, represents a sample of the repertoire that is sequestered in depots (bone marrow, spleen, LN, and tissues). The role of lymphocytes in the circulation has always been postulated to involve purposeful movement from memory depots to lymphoid

TABLE 3 | Coefficients of correlation (R) and determination (R^2) between stability and number of clonotypes for each individual within the study cohorts in reference to **Figure 4**.

Children	**Stability vs. number of clonotypes**	
	R	***R*²**
	Figure 4A	
C1	−0.972	0.945
C2	−0.986	0.972
C3	−0.981	0.962
C4	−0.959	0.920
C5	−0.996	0.992
Average[§]	−0.98 ± 0.01	0.96 ± 0.03
Middle-aged adults	**Figure 4B**	
mA1	−0.928	0.861
mA2	−0.965	0.931
mA5	−0.849	0.721
mA6	−0.817	0.667
Average	−0.89 ± 0.07	0.80 ± 0.12
Older adults	**Figure 4C**	
oA1	−0.902	0.814
oA2	−0.975	0.953
oA3	−0.989	0.979
oA4	−0.898	0.806
oA5	−0.751	0.563
oA6	−0.822	0.676
Average	−0.89 ± 0.09	0.80 ± 0.16

[§]*Indicates mean ± standard deviation.*

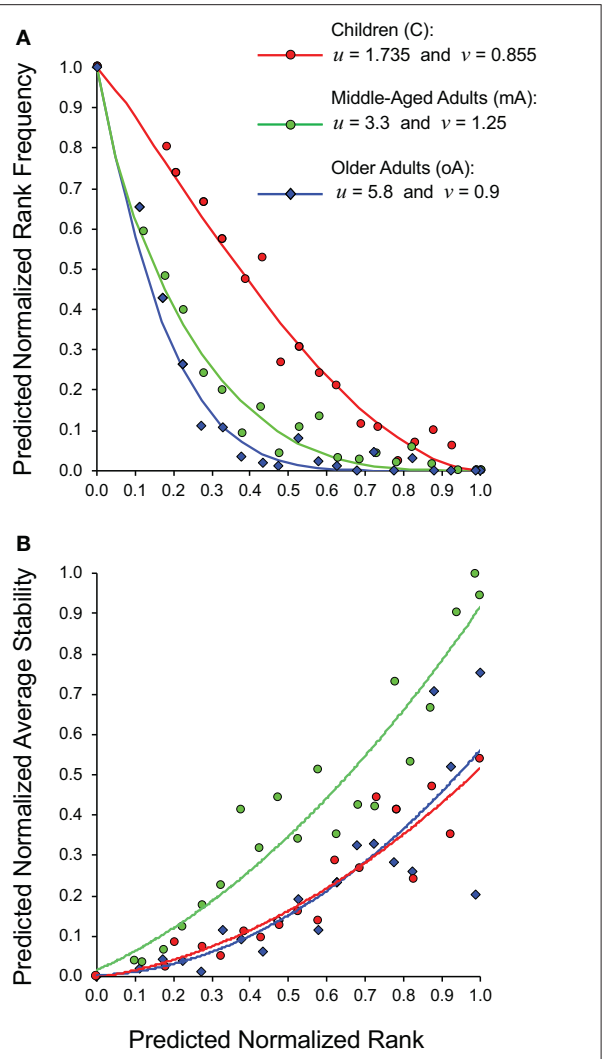

FIGURE 5 | Relationship between estimated clonotype rank, rank frequency and stability of recall BV19 repertoires from child (filled circles), middle-aged adult (open circles) and older adult (diamonds) cohorts. **(A)** Predicted normalized rank vs. predicted normalized rank frequency. **(B)** Predicted normalized rank vs. predicted normalized average stability. Anchored regression lines for children (orange) and middle-aged adults (green) and older adults (blue) are shown.

organs or affected tissues/organs (3, 9, 10). Under normal conditions, only a small portion of circulating CD8 lymphocytes have markers indicating very recent activation (34), most express the inhibitory receptor CD31 (35), hence it is highly likely that they are being released from depots as part of a sentinel process and not in response to an exposure. We assume that the sequestered mature memory repertoire also shows a power law-like distribution. The first component would represent a proportion of the tissue/depot resident repertoire that has entered the circulation. The probability of observing the same clonotype at multiple times would be function of the frequency of that clonotype in the repertoire (its rank) and of the circulatory dwell time.

The second frequency component is over-selected in the analysis process because the continuous presence of these clonotypes in the circulation results in their being sampled at an entirely different frequency than that of the clonotypes in the more dynamic component. Clonotypes in the more stable component have been the focus of previous longitudinal HTS analyses (36, 37). With the presence of two components, examining pooled repertoires would quickly establish the stable portion but would also begin to describe the hidden portion in depots, as a cumulative sum of the dynamic, power law-like component. Ignoring or filtering out this dynamic component would provide an incomplete description of the entire repertoire.

Our data does not rule out the possibility of a second more highly expanding component that could be generated in the initial immune response. It is possible that T cell subsets each have their own reward function as described in **Figure 1**. There is some evidence for this possibility in the child cohort data which shows a hint of an unstable second component (**Figure 5**).

Our focus on canonical BV19 RS-encoding CDR3 length 11 clonotypes, does not rule out the presence of other clonotypes in children, which are supplanted by the canonical clonotypes.

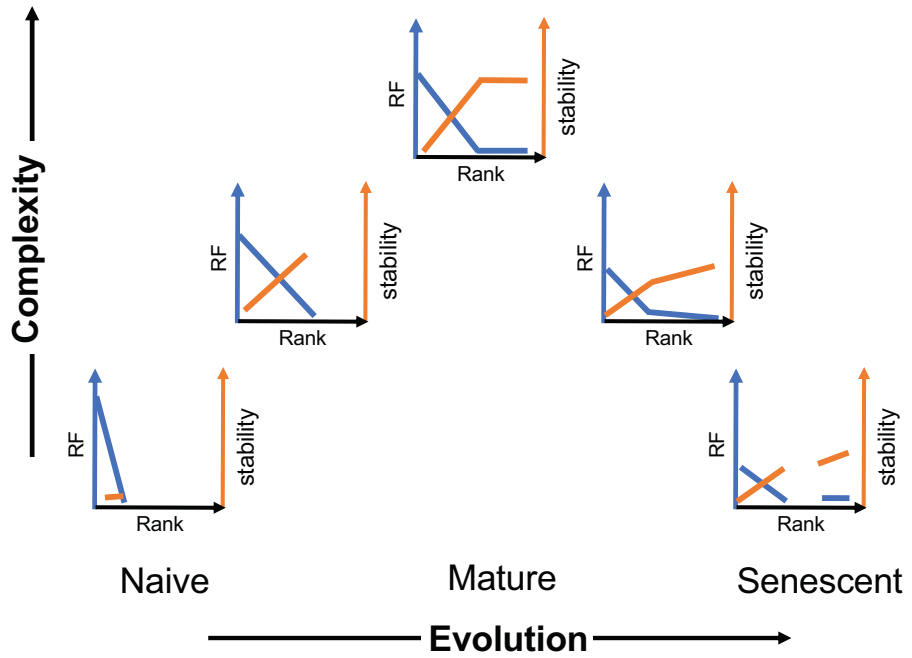

FIGURE 6 | Schematic of the evolution and devolution of repertoire complexity. The y-axis represents complexity entailing both clonotype distribution (blue) and stability (orange). The extent of the complexity is representative and reflects maximum distributional and stability measures at maturity.

T cells responding to $M1_{58-66}$ have been identified in cord-blood and blood from HLA-A2 infants, but these are no longer observed in adults (38, 39).

A circulating pool of stable clonotypes could result from the expansion of important clonotypes beyond the carrying capacity of the memory depots or tissues. We propose it represents maturation stage that maintains a quick response to recurring pathogens. This maintenance would be solidified over time as part of a robust system. It should be pointed out that a circulating depot makes sense for mature effector cells as compared to helper/regulatory T cells. Thus, it will be interesting to determine if cytotoxic CD4 cells which are often observed in mature individuals (40) also have a circulating component. It will also be interesting to define further characteristics of the stable repertoire in terms of the type of pathogen involved, whether chronic or recurring, the tissue dispersion of the pathogen, the degree of cross-reactivity of the T cells, and their surface phenotypes.

Reflection on the nature of a mature robust memory repertoire as well as our dynamic data indicate the importance of repertoire stability. Examining stability as defined here raises important issues about time and sampling, which will require further study. The BV19 *ex vivo* data are representative of short-term stability in that the elapsed time was about a year and a half. The recall data comprised a slightly longer term (**Supplemental Table 1**). The cohort comparisons describe the system over longer elapsed times, but these are not longitudinal. The longer the time span measured longitudinally the more confidence one has in defining a truly stable population. The key points in repertoire maturation are defining when an individual establishes a stable repertoire and when it starts to decay. These measurements are easy for the second distributional component, but more difficult for the first. In addition to frequency of timing, examination of multiple samples per time point would be useful to determine stability as defined by the sampling of a power-law like distribution in comparison to the effect of time. We expect that a careful examination of samples from older children and young adults will show evidence both for a steady buildup of clonotypes that will form the stable circulating pool as well as the transient clonotypes reflecting release from the repertoire depots.

The generation of a stable circulating component is a function of the temporal evolution of the immune system. Stable influenza-specific clonotypes were not observed in the child cohort, appeared to be well-established in middle-aged subjects, and were starting to degrade in older subjects. While our data are focused on one V family and one immune response, the self-similar nature of the system makes it likely that the observed phenomena will carry over to most CD8 T cells and responses.

We present a general schematic of this temporal evolution process for CD8 cells (**Figure 6**) incorporating frequency and stability as a function of rank and moving left to right on a time axis and bottom to top on a complexity axis. A sample of the initial naïve repertoire (lower left panel) would be relatively uniform (mostly rank of one). It would represent a low level of complexity (although high abundance). We have previously examined the frequency of BV19, RS-encoding

clonotypes in CD8 single-positive thymocytes as a proxy for the naïve repertoire, and have shown this to be the case, with a minor skewing due to the function of a rearrangement mechanism involving long P nucleotide addition from the J2-7 region (29). Upon first contacts with influenza the repertoire would show the expansion (increased rank) of selected clonotypes and an increase in stability relative to the frequency of the clonotype in the actual memory depots (second panel). With increasing number of exposures, the repertoire distribution becomes complex but does not develop the stable circulating component until maturity, which would represent the highest level of complexity (middle panel). With time and more exposures, the repertoires become more heterogeneous in their characteristics and both components of the repertoire can devolve independently.

The heterogeneity of an aging immune system is only hinted at in **Figure 6**. We have previously shown that even in middle-age individuals, repertoire changes in a 5- to 10-year time scale, involve a loss of the canonical BV19 RS clonotypes and an increase in other clonotypes utilized in recall responses (41). Our recall data of the older cohort here has been restricted to canonical clonotypes to aid comparison between cohorts. However, we observe a large increase in non-canonical clonotypes in recall responses from older individuals (in preparation). Our previous modeling of the changes between middle- and older-aged adult cohorts indicates an age-related loss of clonotypes based on rank (18). Re-exposure to the virus continues throughout an individual lifetime making it likely that the rank-based loss of complexity observed in the descending part of **Figure 6** is due to such exposures (41). We propose that during the devolution of the repertoire there is an exposure-based loss of clonotypes, compensated by replacement with next best clonotypes, followed by the loss of the compensatory clonotypes, resulting in a tipping point synonymous with immunosenescence. Measuring the individual rate of loss from recurring exposures should provide a warning of immunosenescence and approaching criticality.

Our results describe a dynamic process of system development and aging, with increasing distributional complexity, leading to a stable circulating component, followed by loss of both complexity and stability. Along with a better understanding of the general aspects of memory generation, maintenance and decline, this study poses some fundamental questions of how well we can potentially measure T cell memory in humans and/or how complete this knowledge could be. We still have no answers to how frequently and for how long we should measure a repertoire in order to define its stability. Could a routine blood sample be a representative sample of the circulating pool? And if not, what is the alternative. We expect that stability will be affected by pathogen exposure, hence our care in trying to eliminate that aspect from the current analysis. But what degree of departure from stability would be considered as a measure of resilience or decline? These emergent questions are immediately important in thinking about circulating cells as a source for immunomodulatory therapy and they shape a new direction in quantification of the way immune memory evolves.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher. The curated dataset used for the analyses here are provided as **Supplemental Data 1**.

ETHICS STATEMENT

The healthy child subjects were enrolled under protocol CHW IRBnet: 116305 Generation and decay of memory T cells in children with Juvenile Rheumatoid Arthritis and healthy siblings following administration of trivalent inactivated influenza vaccine, from the Children Hospital of Wisconsin. The subjects analyzed here were the controls in this study. Written informed consent was obtained from participants, or their parents/legal guardians in the case of children. The adult subjects were enrolled under protocols authorized by the Institutional Review Board of BloodCenter of Wisconsin: BC 05-11, Generation and Decay of Memory T Cells in Older Populations, and BC 04-22, Robust T Cell Immunity to Influenza in Human Populations. These protocols have been transferred to the IRB of the Medical College of Wisconsin (MCW).

AUTHOR CONTRIBUTIONS

All authors have read and approved the manuscript. EN implemented and performed the high-level analyses and participated in writing the paper. MY was involved in both the *ex vivo* and recall analyses, and in organizing the experiments. WD was involved in the *ex vivo* analyses. ER and MU generated the recall data for the adult cohorts. DH generated the recall repertoire and MU analyzed the clonotypes in the child cohort. CW had overall responsibility for the child cohort analyses. YN was involved in analyzing the recall repertoire in the adults, and in data analysis. JG was responsible for the overall study design and writing the paper.

FUNDING

This work supported by NO1 AI50032 (JG) and U19 AI062627 (JG).

ACKNOWLEDGMENTS

We thank Dr. Liz Worthy and Mike Tschannen at the Human and Molecular Genetics Center of the Medical College of Wisconsin for Roche 454 sequencing. We also thank Lucy Stewart, Amalia Corby-Edwards, and Marsha Malloy for coordinating the studies from which the samples were drawn, and Lee Fong and Va Xiong for PBMC preparation, characterization, and storage.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.01717/full#supplementary-material

Supplemental Figure 1 | Clonotype naming algorithm. The name is meant to be available for computation. It consists of the concatenation of the NDN amino acids

in single letter code, in upper case, proceeded and followed by the last amino acid completely V-encoded and the first completely J encoded in lower case. A period separates the amino acids from the NDN encoding. This is followed by the V identifier which starts with B or A for beta or alpha followed by the family number, the letter "s" and the subfamily identifier. Next come the J-region identification, with again A for alpha, B for beta. Followed by the J cluster and locus position identifier, with no separator. The name provides the information as to the key diversity element that distinguishes this V-J combination from any other using the exact same V and J. To regenerate the CDR3 nucleotide sequence from the name one moves backwards through the naming algorithm. **(A)** We start with the clonotype name of the most frequent clonotype in the C1 recall dataset. The key data are colored to identify their content. **(B)** The genomic sequences of the V and J are lined up so that there are L codons between the Cys and Phe Gly. The Amino acids of the NDN (upper case) are aligned and the nucleotide sequence of each is inserted. **(C)** The nucleotide sequence is assembled by overlapping the V, NDN, and J sequences with the V and J lower case base pairs being used first until they no longer match the NDN codons. The germline contribution to the CDR3 is underlined in the sequences provided in **(B)**. This provides the best estimate of the rearrangement point. **(D)** The subset of the genetic code table needed for converting the a.a. name and encoding to nucleotide sequence in step B. The period in the name provides a visual break, but is also useful to FIND the start of the codon ID string. The NDN region can be substringed by starting at position 2 and stopping before the period. The NDN length can be determined from the same procedure. The "L" provides a visual break between the J and CDR3 length numerical identification. The letter "S" was used at one time to separate the V family and Subfamily identification. Current nomenclature uses a bar. Current nomenclature does not require a TRBV19-1 since there is no 19-2 locus. However we include the subfamily tag to maintain spacing in the name. All lengths take two characters, hence a CDR3 length of 9 amino acids is L09. Alleles are neglected. There are no V alleles reported yet in the region distal to the Cys. There are two J-region alleles, one is silent, but the one in J2-7 changes the F in the FG to V. The nomenclature could be modified to include allele identification but it would be wise to avoid the current nomenclature use of an asterisk as it is usually interpreted as a wild card and requires additional handling if the name is being used in a computation.

Supplemental Table 1 | Time-map of peripheral blood collection for each subject relative to first sampling.

Supplemental Table 2 | Summary of high-throughput sequencing data for the adult cohort.

Supplemental Table 3 | Measures and characteristics of the M_{158-66}-specific recall repertoires for the child cohort.

Supplemental Table 4 | Measures and characteristics of the M_{158-66}-specific recall repertoires for the middle-aged adult cohort.

Supplemental Table 5 | Measures and characteristics of the M_{158-66}-specific recall repertoires for the older adult cohort.

Supplemental Table 6 | Summary of M_{158-66}-specific recall BV19 repertoire measures and characteristics for the child, middle-aged, and older adult cohorts.

Supplemental Data 1 | The clonotype names, counts, and stability values for all the analyses presented in this manuscript are provided.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 07 August 2019
doi: 10.3389/fimmu.2019.01888

Different Expression Characteristics of LAG3 and PD-1 in Sepsis and Their Synergistic Effect on T Cell Exhaustion: A New Strategy for Immune Checkpoint Blockade

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Specialty section:
This article was submitted to Immunological Memory, a section of the journal Frontiers in Immunology

Received: 30 May 2019
Accepted: 25 July 2019
Published: 07 August 2019

Citation:
Niu B, Zhou F, Su Y, Wang L, Xu Y, Yi Z, Wu Y, Du H and Ren G (2019) Different Expression Characteristics of LAG3 and PD-1 in Sepsis and Their Synergistic Effect on T Cell Exhaustion: A New Strategy for Immune Checkpoint Blockade. Front. Immunol. 10:1888. doi: 10.3389/fimmu.2019.01888

The impairment of immunity characterized by T cell exhaustion is the main cause of death in patients with sepsis after the acute phase. Although PD-1 blockade is highly touted as a promising treatment for improving prognosis, the role of PD-1 plays in sepsis and particularly its different roles in different periods are still very limited. A recent study revealed LAG3 can resist the therapeutic effect of PD-1 blockade in tumor, which inspired us to understand their role in sepsis. We enrolled 26 patients with acute sepsis from 422 candidates using strict inclusion criteria. Follow-up analysis revealed that the expression levels of PD-1 were rapidly increased in the early stage of sepsis but did not change significantly as infection continued ($P < 0.05$). However, the expression of LAG3 was contrary to that of PD-1. Compared with LAG3 or PD-1 single-positive T cells, T cells coexpressing LAG3 and PD-1 were significantly exhausted ($P < 0.05$). The proportion of coexpressing T cells was negatively correlated with the total number of lymphocytes ($r = -0.653$, $P = 0.0003$) and positively correlated with the SOFA score ($r = 0.712$, $P < 0.0001$). In addition, the higher the proportion of coexpressing T cells was, the longer the hospital stay and the higher the mortality. These results showed that LAG3 and PD-1 had a potential synergistic effect in regulating the gradual exhaustion of T cells in sepsis, which seriously affected the clinical prognosis of patients. Therefore, LAG3 and PD-1 double-positive T cells are an important indicator for immunity detection and prognostic evaluation. In the future, precision therapy may pay more attention to the different expression patterns of these two molecules.

Keywords: T cell exhaustion, sepsis, PD-1, LAG3, synergistic inhibition

HIGHLIGHTS

- In sepsis, LAG3 and PD-1 had unique expression characteristics in T cells, and the T cells that coexpress LAG3 and PD-1 were significantly exhausted.
- The proportion of T cells coexpressing LAG3 and PD-1 was negatively correlated with the total number of lymphocytes and positively correlated with the SOFA score.
- In septic patients, the higher the proportion of LAG3 and PD-1-coexpressing T cells was, the longer the hospital stay and the higher the mortality.

INTRODUCTION

Sepsis is characterized by an intense systemic response to infection. The incidence rate is estimated to be up to 30 million cases and 6 million deaths worldwide per year, and the number of cases is rising (1) and has become the leading cause of death in intensive care units (2, 3). The pathogenesis of sepsis is the result of a complex network of events involving proinflammatory and anti-inflammatory processes triggered by the infectious agent (4). Postmortem studies of patients who died of sepsis have provided important insights into why septic patients die and highlighted key immunological defects that impair host immunity (5, 6). One of the most important features of immunosuppression is T cell exhaustion (7, 8). Many factors are involved in this process, and negative costimulatory molecules are considered to be the very important elements (5, 8–11). Recently, some negative costimulatory molecules have shown interactive relationships in non-septic disease, and these relationships seriously affect the occurrence and development of disease, particularly the relationship between lymphocyte-activation gene 3 (LAG3) and programmed cell death 1 (PD-1) (12–15). In particular, a recent study showed that the activation of LAG3 can resist the efficacy of anti-PD-1/B7-H1 therapy in tumor (16), and dual blockade of LAG3 and PD-1 can provoke more powerful antitumor or antiviral effects than the sum of blocking each molecule alone (12, 13, 15, 17–20). However, whether they also interact in sepsis which is different from the chronic pathological changes mentioned above has not been studied. Here, we performed a prospective observational study and systematically analyzed the expression characteristics and functions of LAG3 and PD-1 in T cells as well as the relationship between LAG3 and PD-1 and the prognosis of patients with sepsis.

Abbreviations: PD-1, Programmed cell death 1; LAG3, Lymphocyte-activation gene 3; AECOPD, Acute Exacerbation of Chronic Obstructive Pulmonary Disease; ICU, Intensive Care Unit; SOFA, Sequential [Sepsis-related] Organ Failure Assessment; APACHE II, Acute Physiology and Chronic Health Evaluation II; HIV, Human Immunodeficiency Virus; RPMI, Roswell Park Memorial Institute; EDTA-2Na, disodium ethylenediaminetetraacetate dihydrate; FBS, Fetal Bovine Serum; BSA, Bull Serum Albumin; MESF, Molecules of Equivalent Soluble Fluorochrome; PBS, Phosphate Buffer Saline; FSC, Forward Scatter; SSC, Side Scatter; ELISA, Enzyme-linked immuno sorbent assay; IL-2, human interleukin-2; IL-6, human interleukin-6; TNF-α, tumor necrosis factor-alpha; IFN-γ, interferon-gamma.

MATERIALS AND METHODS

Patients' Enrollment and Clinical Data Collection

Adult patients with suspected infection from the emergency department admitted to the medical and surgical ICU at the First Affiliated Hospital of Chongqing Medical University were followed up and screened for sepsis daily, using the sepsis 3.0 criteria (21), and the organ damage was assessed via the sequential [sepsis-related] organ failure assessment (SOFA) score (22). All patients needed to meet the criteria of sepsis 3.0 upon enrollment. Patients with end-stage chronic diseases, such as uremia and liver failure, active malignancy, death within 48 h, or chronic viral infection, such as HIV, hepatitis B or C; taking immunosuppressive medications with corticosteroids at doses $\geq$10 mg prednisone or equivalent per day (23); or diagnosed with other diseases that could also affect host immunity, as shown in **Figure 1**, were excluded. The control subjects consisted of age- and sex-matched non-septic critical patients with the same APACHE II scores to study group, and the main diseases were stoke, myocardial infarction or acute intoxication, and none of them had any of the immunocompromised diseases mentioned above. Details of the sepsis patient and control subjects are shown in **Figure 1** and **Table 1**. Other relevant clinical data were also collected, mainly including average hospital stay, mortality rate, absolute number of peripheral blood lymphocytes and SOFA score related indicators. Informed consent is required and obtained from the legally authorized patient representatives, due to all patients admitted to the ICU were judged to be too seriously ill to provide valid consent.

Sample Collection and Primary Treatment

About 5 ml blood with disodium ethylenediaminetetraacetate dihydrate (EDTA-2Na) anticoagulation was collected through an indwelling central venous catheter or venipuncture on day 1 and again on day 5. The blood was instantly processed in our laboratory. Peripheral blood mononuclear cells (PBMCs) were isolated via Ficoll-Hypaque density gradient centrifugation following standard protocols. The cells were washed and resuspended in T cell medium (Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin and streptomycin at an active concentration of 100 units per milliliter each, L-glutamine and non-essential amino acids) and processed for flow cytometry, proliferation or cytokine secretion evaluations as described below.

Antibodies and Reagents

All flow cytometry antibodies were purchased from BD Pharmingen (San Diego, CA, USA), BioLegend (San Diego, CA, USA) and KeyGen Biotech (Nanjin, Jiangsu, China). The following antibodies were obtained from BD Pharmingen^{TM}: CD3-APC-Cy^{TM}7, CD4-FITC, CD8-PECy^{TM}5, PD-1-APC, and LAG3-PE. The following reagents were obtained from BioLegend: PerCP/Cy5.5-conjugated anti-IL-2, PE/Cy7-conjugated anti-IL-6, PerCP/Cy5.5-conjugated anti-TNF-α, and PE/Cy7-conjugated anti-IFN-γ antibodies, a FITC Annexin V/PI

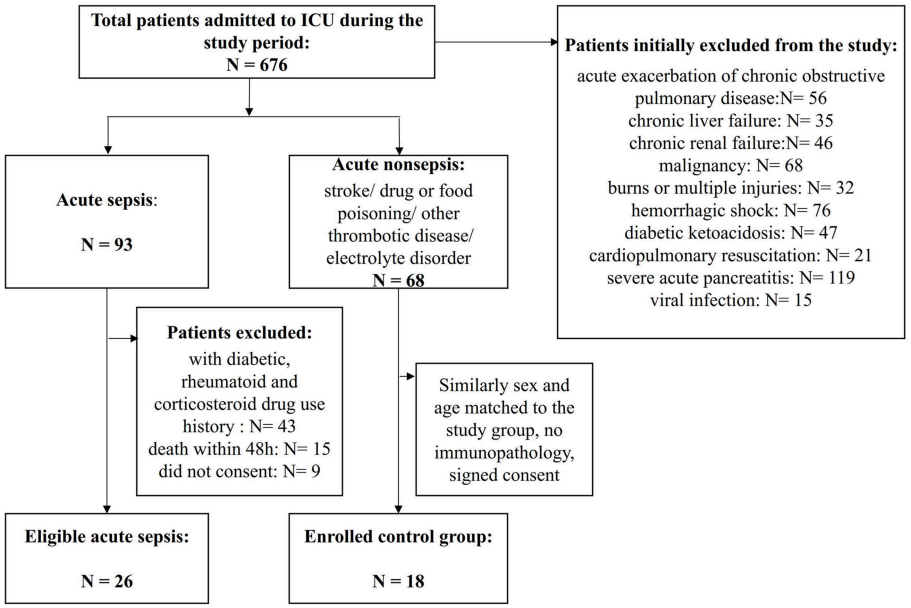

FIGURE 1 | Flowchart of patient enrollment. A total of 676 patients were admitted to the ICU during the study period, and among them, 422 patients were identified as having severe infection. However, many patients had associated chronic immunocompromising diseases or drug use or acute exacerbations of chronic infections and needed to be excluded. Finally, 26 patients with sepsis were enrolled, and 18 patients with similar critical scores that were sex- and age-matched non-septic patients were enrolled as the controls.

kit, and a KGA: FITC-BrdU kit. The following quantum MESF beads were purchased from Bangs Laboratories: Fluorescent Microspheres, Intensity Standard: Dragon Green, Flash Red, PE-MESF, and APC-MESF. The PMA/ionomycin mixture (250X) was purchased from MultiSciences (Lianke) Biotech (Hangzhou, Zhejiang, China). Enzyme-linked immuno sorbent assay (ELISA) kits for human interleukin-2 (IL-2), IL-6, tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) were purchased from Beijing 4A Biotech (Beijing, China). Brefeldin A was purchased from Qcbio Science & Technologies Co., Ltd. (Beijing, China).

Flow Cytometry, Immunofluorescence, Proliferation, and Cytokine Analysis

Target cells were collected at various time points and stained with the appropriate antibodies. The samples were run on a FACS flow cytometer (BD Biosciences) and analyzed by FCS Express. For surface marker staining, 100 μl of PBMCs was incubated with 20 μl of human AB serum and the indicated fluorescently conjugated antibodies for 1 h at room temperature. The expression levels of LAG3 and PD-1 were observed by laser-focused microscopy. Then, the cells were extensively washed in PBS with 1% bovine serum albumin (BSA) and resuspended in PBS with 2% BSA and 2% paraformaldehyde (PFA). Viable lymphocytes were identified and gated by forward scatter (FSC) and side scatter (SSC) properties. T cells were identified as $CD3^+$, with subtypes of $CD4^+$ T and $CD8^+$ T cells. For proliferative and cytokine secretion function detection, PD-1^-LAG3^-, PD-1^+LAG3^-, PD-1^-LAG3^+, and PD-1^+LAG3^+ cells were sorted to more than 90% purity by FACS, labeled with CFSE (2 μM) for 10 min, cultured in 24-well plates, activated by α-CD3/α-CD28, and stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) for 48 h. The culture supernatant was harvested at 12 and 48 h following stimulation, and cytokine levels were determined using ELISA kits according to the manufacturer's instructions. We measured human IL-2 and IL-6 levels for $CD4^+$ T cells and TNF-α and IFN-γ levels for $CD8^+$ T cells. Furthermore, we also determined the amounts of intracellular cytokines that were synthesized but not secreted via flow cytometry. Briefly, after the stimulation described above, Brefeldin A, an intracellular protein transport inhibitor, was added into the culture system. Forty-eight hours later, the T cells were collected, fixed with 4% paraformaldehyde, treated with 0.1% Triton-100 and flow cytometry antibodies, and then analyzed by flow cytometry.

Laboratory Fluorescence Quantitation

In order to quantitatively detect the expression level of related receptors, quantum MESF beads were run with each flow cytometric assay. The Quantum beads are microspheres that each has a fixed fluorescence. The corresponding fluorescence peaks were obtained when the microspheres were run on a flow cytometer to provide individual peaks (six peaks for FITC and five peaks for PE). These peaks of known fluorescence intensities were converted to Molecules of Equivalent Soluble Fluorochrome (MESF) units using QuickCal^{TM} software to generate a standard curve. The mean fluorescence intensity (MFI) of each marker was converted to MESF units based on the Quantum Bead MESF standard curve, according to a previously described method (25, 26).

TABLE 1 | Clinical characteristics of patients with sepsis and controls.

Parameter	Patients with sepsis ($n = 26$)	Control subjects ($n = 18$)	*P*-value
Males, percentage (number)	61.5 (16)	61.1 (11)	0.382
Age, years (range)	50 (32 to 78)	53 (38 to 82)	0.057
SOFA score at admission, median (range)	7.6 (3 to 16)	5.9 (3 to 13)	0.055
APACHE II score at admission, median (range)	16.5 (12 to 32)	14.7 (8 to 24)	0.062
28-day mortality, number (percentage)	5 (19.23)	4 (22.22)	0.217
Ventilation days, median days (range)	5.5 (2 to 17)	9.6 (2 to 26)	<0.001
Length of ICU stay, median days (range)	7.5 (3 to 22)	9.2 (4 to 32)	0.052
Length of hospitalization, median days (range)	16.4 (7 to 36)	25.6 (6 to 89)	0.008
White blood cell at intake, mean ($\times 10^9$/L) (range)	13.66 (2.8 to 38.5)	10.12 (5.8 to 14.5)	0.032
Absolute lymphocyte count, median ($\times 10^9$/L) (range)	0.71 (0.28 to 1.22)	1.42 (0.98 to 2.31)	<0.001
Procalcitonin, median (nanogram /L) (range)	45.5 (10 to 286)	0.32 (0.05 to 1.32)	<0.001
C-reactive protein, median (milligram /L) (range)	82.4 (42 to 142)	47.5 (22 to 92)	<0.001
Shock*, number (percentage)	20 (76.9)	1 (5.56)	<0.001
Biliary tract infection, number (percentage)	6 (23.1)	n/a	n/a
Urinary system infection, number (percentage)	9 (34.6)	n/a	n/a
Pelvic and abdominal cavity infection, number (percentage)	4 (15.4)	n/a	n/a
Other site infection, number (percentage)	7 (26.9)	n/a	n/a

APACHE II, Acute Physiology and Chronic Health Evaluation II; SOFA, Sequential [Sepsis-related] Organ Failure Assessment; n/a, Not Applicable; Shock, the patients with septic shock can be clinically identified by a vasopressor requirement to maintain a mean arterial pressure of 65 mm Hg or greater and serum lactate level >2 mmol/L (>18 mg/dL) in the absence of hypovolemia (24).*

Statistical Analysis

Data were analyzed with the statistical software Prism version 7 (GraphPad, San Diego, CA, USA), and expressed as the mean ± SEM or shown as a box plot. For comparisons of two groups, Student's *t*-test was employed. One-way ANOVA with Tukey's multiple comparison test was used to analyze data containing more than two groups. For survival studies, a log-rank test was used. Two-tailed non-parametric Wilcoxon matched pairs test, two-tailed Mann-Whitney U test and the Kruskal-Wallis test were used for non-parametric data. To test for correlations, Pearson's simple correlation coefficient was applied. $P < 0.05$ were considered to indicate statistically significant differences.

RESULTS

Patient Enrollment and Specimen and Clinical Data Collection

A flowchart of patient enrollment is shown in **Figure 1**. Ultimately, 26 subjects with acute sepsis and 18 control subjects were included in the study. The clinical characteristics of the patients with sepsis and controls are displayed in **Table 1**. There were no significant differences in gender, age, SOFA scores and APACHE II scores, 28-day mortality, and length of ICU stay between the two groups, and the *P*-values were 0.382, 0.057, 0.55, 0.062, 0.217, and 0.052, respectively. However, the differences in the WBC, ventilation days, and length of hospitalization, procalcitonin, C–reactive protein, and the percentage of shock patient between the two groups were conspicuous ($P < 0.05$). The absolute number of lymphocytes decreased more significantly in the sepsis group ($P < 0.001$).

Expression Characteristics of PD-1 and LAG3 in Peripheral T Cells During the Onset of Sepsis

The patients admitted to hospital with infection were screened daily for sepsis, and the moment when they reached the sepsis criteria was identified as onset of sepsis. To determine the number of lymphocytes and the expression of LAG3 and PD-1 on $CD4^+$ T and $CD8^+$ T lymphocyte surfaces, blood was obtained within 12 h of the onset of sepsis and extensively characterized by flow cytometry. An identical analysis was performed on the non-septic control subjects to provide comparative data. In the patients in the acute phase of sepsis, the expression of PD-1 on both $CD4^+$ T cells and $CD8^+$ T cells was significantly elevated compared with that in the controls ($P < 0.05$) (**Figure 2**). However, the expression of LAG3 on $CD4^+$ T cells or $CD8^+$ T cells was not obviously elevated in the sepsis group compared with the control group, and the *P*-values were 0.28 and 0.19 for the expression rate (%) and expression intensity (MESF) of $CD4^+$ T cells, respectively, and 0.32 and 0.17 for the expression rate (%) and MESF of $CD8^+$ T cells, respectively (**Figure 2**).

Changes in PD-1 and LAG3 Expression Over the Course of Acute Sepsis

Blood was collected from the patients and controls on the 5th day and analyzed. As presented in **Figure 3**, compared with the control group, the sepsis group exhibited obviously higher PD-1 expression levels on both $CD4^+$ and $CD8^+$ T cells ($P < 0.05$). In addition, the LAG3 expression levels were also distinctly elevated on both $CD4^+$ and $CD8^+$ T cells ($P < 0.05$) (**Figures 3A,B**). We separately compared the changes in PD-1 and LAG3 expression on $CD4^+$ and $CD8^+$ T cells in the septic patients. Interestingly,

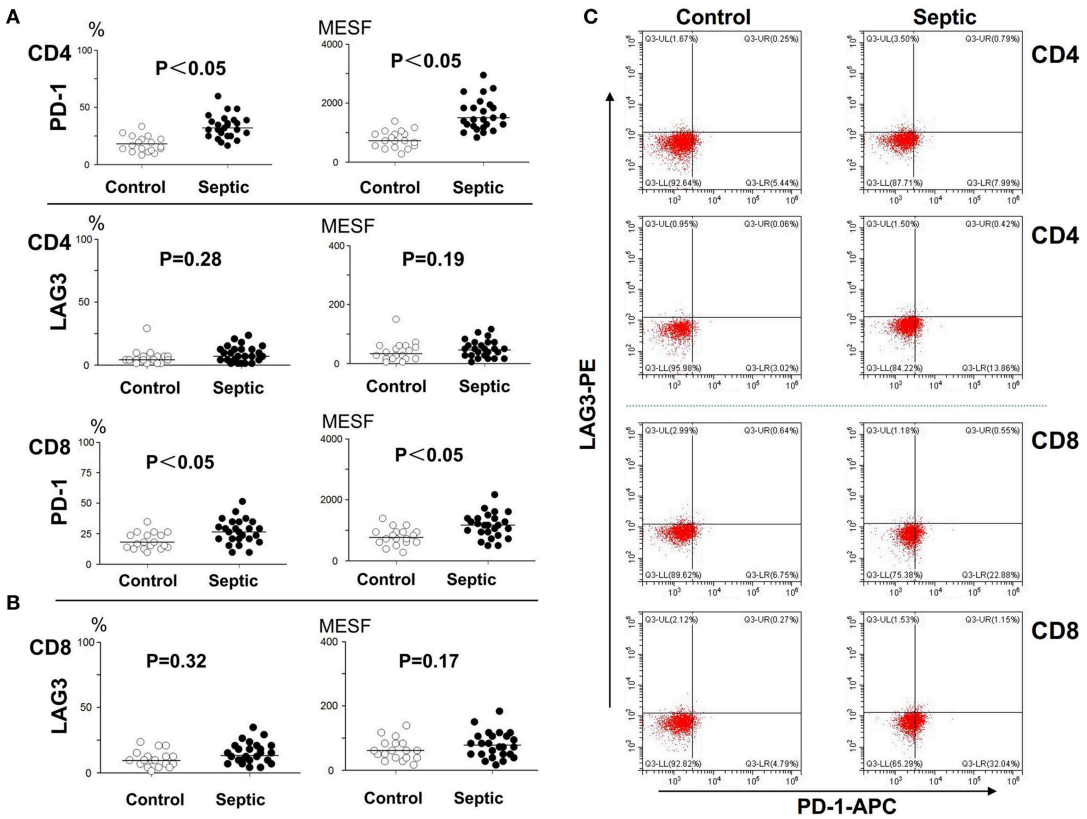

FIGURE 2 | Determination of the expression of LAG3 and PD-1 on CD4 and CD8 T cells at the onset of sepsis. The peripheral blood of septic patients and controls were collected within 12 h after enrollment, and then stained for flow cytometry. The positive rate relative to that of the isotype control staining was determined. Fluorescence intensity was quantified by calculating MESF values as described in the procedures. The horizontal lines represent the mean levels. Scattergram **(A)** and **(B)** showed the expression of LAG3 and PD-1 on CD4 and CD8 Tcells, respectively. Representative flow cytometry pictures **(C)**. *P*-values were calculated with a non-parametric 2-tailed Mann-Whitney U test.

there were no significant differences in the expression of PD-1 on $CD4^+$ or $CD8^+$ T cells between the day of onset and the 5th day of sepsis ($P > 0.05$) (**Figures 4A,B**). Nonetheless, LAG3 had a trend toward an increase in the expression rate (%) ($P < 0.05$) and per cell intensity (MESF) ($P < 0.05$) from day 1 to day 5 in both $CD4^+$ and $CD8^+$ T cells (**Figures 4A,B**).

Co-expression of PD-1 and LAG3 on T Cells in the Extended Phase of Sepsis

As mentioned above, we analyzed the negative costimulatory molecules PD-1 and LAG3 to determine the expression and changes in these molecules in both $CD4^+$ and $CD8^+$ T cells. Here, we further analyzed their coexpression on the same $CD4^+$ and $CD8^+$ T cells collected on the 5th day of sepsis. The PD-1 and LAG3 coexpression rates were significantly elevated for both the $CD4^+$ and $CD8^+$ T cells from the septic patients compared with those from the controls ($^*P < 0.05$;$^{**}P < 0.05$) (**Figure 5A**); however, the proportion of coexpressing $CD8^+$ T cells was significantly higher than that of coexpressing $CD4^+$ T cells ($^{***}P < 0.05$) (**Figure 5B**). By immunofluorescence, the expression of LAG3 and PD-1 on the surface of T cells on the 5th day of sepsis was also directly observed (**Figure 5C**).

Stimulated T Cells With Different Phenotypes of Cytokine Secretion, Proliferation, and Apoptosis in Patients With Sepsis

We conducted cell sorting for cells with different phenotypes in the peripheral blood of the patients with sepsis and controls on the 5th day after enrollment through flow cytometry. After standardizing the concentrations, the different types of T cells including $PD\text{-}1^+LAG3^-$ T cells, $PD\text{-}1^-LAG3^+$ T cells, $PD\text{-}1^+LAG3^+$ T cells and control T cells were cultured in 24-well plates at the same concentration and then activated and stimulated for 48 h. We found that the levels of IL-2 and IL-6 mainly secreted by $CD4^+$ T cells and IFN-γ and TNF-α mainly secreted by $CD8^+$ T cells were lowest in $PD\text{-}1^+LAG3^+$ T cells groups (**Figures 6A–D**), and regardless of the terminal concentration or the rate of secretion increase, the levels of these factors were all significantly lower in the $PD\text{-}1^+LAG3^+$ T cell group than in the other three groups. We also determined the

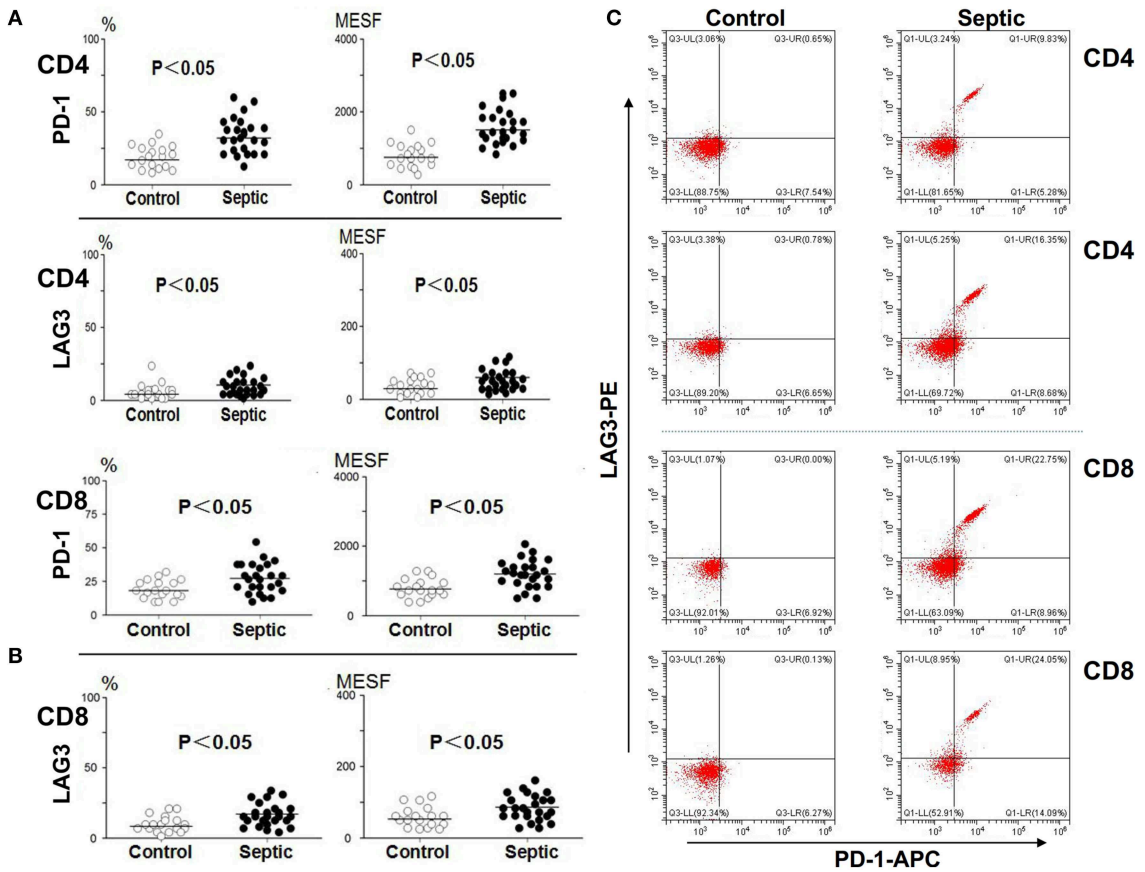

FIGURE 3 | The expression of LAG3 and PD-1 on CD4 and CD8 T on the 5th day of the sepsis. The peripheral blood of septic patients and controls were collected at enrollment and again 5 days later, and analyzed by flow cytometry for the expression of the indicated markers on CD4 T cells and CD8 T cells. The positive proportion and MESF values were obtained as described in the procedures. The horizontal lines represent the mean levels. Scattergram **(A)** and **(B)** showed the expression of LAG3 and PD-1 on CD4 and CD8 Tcells, respectively. Representative flow cytometry pictures **(C)**. *P*-values were calculated with a non-parametric 2-tailed Mann-Whitney U test.

intracellular levels of these cytokines in the different groups. The intracellular cytokine levels and extracellular supernatant cytokine concentrations showed the same trends, as displayed in **Figures 6E–H**. Moreover, the levels of these cytokines were extremely low in the PD-1^{+}LAG3^{+} T cell groups compared with the other groups. Based on the detection of apoptosis, we found that the total (early and late) apoptosis rates were most increased in the PD-1^{+}LAG3^{+} T cell groups compared with the other groups (**Figure 7B**). As for proliferative function, we used CFSE staining for cell division analysis (**Figures 8A–G**) and FITC-BrdU staining for proliferation rate determination (**Figure 8G**). After activation and stimulation, the T cells of the control groups rapidly divided and proliferated; only approximately 12% of the parental cells could be detected, and the seventh generation cells accounted for approximately 65% (**Figure 8A**). In contrast, the PD-1^{+}LAG3^{+} T cells showed very slow division and proliferation rates, with more than 80% of the parental cells not undergoing division or proliferation, and seventh generation cells were barely detectable (**Figure 8D**). Although the proliferative functions of the PD-1^{-}LAG3^{+} T cells and PD-1^{+}LAG3^{-} T cells were damaged, their proliferative capacities were still significantly higher than those of the PD-1^{+}LAG3^{+}T cells (**Figures 8A–G**). Furthermore, we also found that the absolute number of lymphocytes in the patients with sepsis was negatively correlated with the proportion of LAG3 and PD-1 double-positive T cells ($r = -0.653$, 95%CI: -0.831 to -0.356, $P = 0.0003$) (**Figure 8H**).

Statistical Analysis of Other Clinical Data: The SOFA Score, Hospitalization Days and Mortality

Previously, we showed that LAG3 and PD-1 double-positive T cells are significantly impaired in terms of proliferation and antiapoptosis function and that the higher the proportion of double-positive T cells is, the lower the absolute number of lymphocytes in patients. Do these cells have any influence on other clinical indicators in patients with sepsis? Here, the SOFA score, hospitalization days and mortality were analyzed. We found that the proportion of LAG3 and PD-1 double positive T cells was positively correlated with the SOFA score ($r = 0.712$, 95%CI: 0.448 to 0.862, $P < 0.0001$) (**Figure 9A**). To

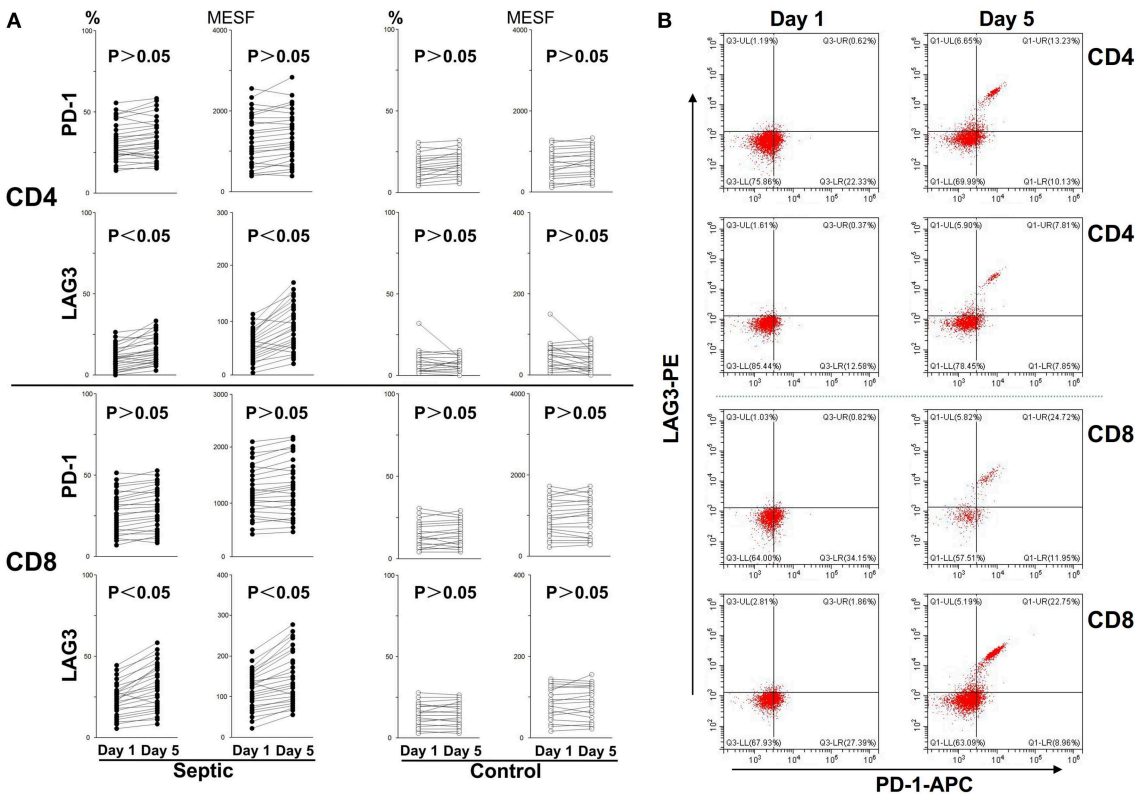

FIGURE 4 | Changes in LAG3 and PD-1 expression on CD4 and CD8 T cells over the course of acute sepsis. The lines connect values at enrollment and the 5th day after the onset of sepsis or on the 5th day in the control group. The positive proportion and MESF values were obtained as described in the procedures. Scattergram **(A)** showed the expression changes of LAG3 and PD-1 on CD4 and CD8 Tcells, respectively. Representative flow cytometry pictures of sepsis group from day 1 to day 5 **(B)**. As there were no significant changes in the control group from day 1 to day 5, we did not present representative images. *P*-values were calculated with a 2-tailed, non-parametric Wilcoxon matched pairs test.

understand the relationship between the proportion of LAG3 and PD-1 double-positive T cells and the length of hospital stay, mortality or overall survival, we stratified the proportions at 5% intervals including 1–5%, 6–10%, 11–15%, 16–20%, 21–25%, and no subject had a proportion above 25%. **Figure 9B** shows that the higher the double-positive proportion was, the longer the hospital stay, and there were significant differences among the proportion ranges. Similar trends were observed for mortality and overall survival, specifically, the higher the proportion range was, the higher the death rate and lower the survival (**Figure 9C**).

DISCUSSION

Sepsis, a systemic inflammatory condition due to severe infection, has become the most common cause of mortality in most intensive care units (27–29). Improved treatment protocols and updated organ support equipment have resulted in the majority of patients surviving the initial 72 h of sepsis only to succumb later in the time course of the disease (30, 31). The failure of several high-profile clinical trials in sepsis has led basic and clinical researchers to state that sepsis studies need a new direction, and there is increasing recognition that a state of impaired immunity follows the initial hyperinflammatory phase of sepsis (8, 10, 27, 32). An important feature of immunosuppression is T cell exhaustion, which was recently recognized following many trials for sepsis involving immunoregulatory therapies, such as PD-1 blockade, interleukin 7 administration, interleukin 15 administration, IFN-γ administration, and CTLA-4 blockade (2, 21, 31, 33). PD-1 is considered to be one of the most promising targets for immunomodulatory therapy in sepsis (31, 33–35), and further studies have confirmed that anti-PD-1 treatment did not meet expectations in all conditions because multiple negative costimulatory molecules are expressed on the surface of exhausted T cells. Researchers have also passively explored multitarget combination blockades, such as combining anti-PD-1 and anti-CTLA-4 therapies, to maximize the recovery of T cells and obtain better therapeutic effects (31, 34). In fact, our understanding of the roles and mechanisms of these negative costimulatory molecules in sepsis is rather limited. Recently, the potential relationship between PD-1 and LAG3 was reported in other non-septic diseases (12, 15, 17, 18), and moreover, LAG3 activation can resist the therapeutic effect of PD-1 blockade (16), but their role in sepsis is still unclear.

Due to the special pathophysiological status of critically ill patients, such as stress state, adrenal secretion level, nutrient

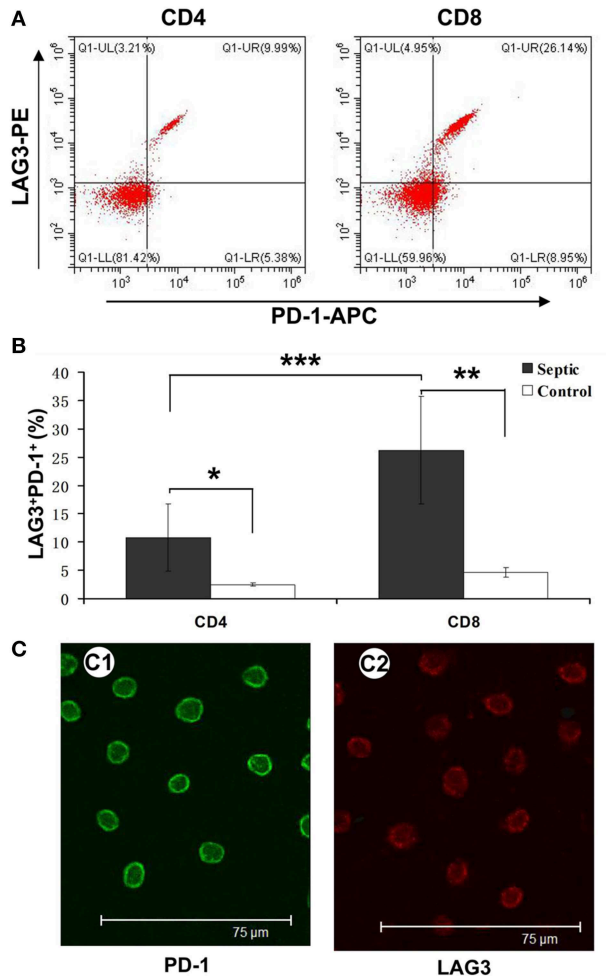

FIGURE 5 | Determination of the coexpression of LAG3 and PD-1 on CD4 and CD8 T cells over the course of acute sepsis. Peripheral blood was collected at enrollment and again 5 days later from patients with sepsis and normal controls. We could directly observe T cells with high expression of LAG3 and PD-1 by immunofluorescence **(C)**. After analysis by flow cytometry for the expression of the indicated markers on CD4- and CD8-positive T cells, the ratio of $CD8^+$ T cells with LAG3 and PD-1 coexpression was close to 25% and higher than that of $CD4^+$ T cells, which was approximately 8% **(A)**; $^{***}P < 0.05$ $CD4^+$ T cells vs. $CD8^+$ T cells in sepsis **(B)**. However, both ratios were significantly higher than those for the control group; $^{*}P < 0.05$ and $^{**}P < 0.05$, respectively **(B)**. Data are presented as mean ± SEM, Student's *t*-test was employed.

intake, and so on, were differently from those of healthy volunteers. In addition, there has been much research on comparing the sepsis with healthy volunteers (5, 8, 10, 23, 31). In particular, it needs to be emphasized, the expression of LAG3 and PD-1 on the surface of T cells of healthy volunteers will not change significantly with time goes on (23, 31). In order to reduce baseline differences in immune status, as much as possible, between critically ill patients and controls, and to highlight the expression changes of negative costimulatory molecules in T cells of septic patients, we designed this study to compare non-septic and septic critically ill patients without comparing with healthy volunteers. It should be noted that this pairwise group study was only used to compare the dynamic changes of negative costimulatory molecules, and did not involve functional analysis of T cells with different phenotypes and their clinical prognostic effects. Therefore, this study does not need to compare with the healthy control group, and its conclusions are not biased. As the control subjects, the non-septic population was selected to be approximately age and sex matched with the septic population. There was no significant difference in the degree of critical illness between the two groups, either in the SOFA scores ($P > 0.05$) or in the APACHE II scores ($P > 0.05$). More importantly, we used strict exclusion criteria; all other known diseases and a history of medication that could affect host immunity were excluded. We finally enrolled 26 subjects and 18 controls from 676 candidates, and many of those excluded patients met the sepsis criteria, might be included in previous other sepsis studies, but those patients themselves more or less combined with immune damage factors and could not really reflect T-cell function. Although the inclusion of patients was relatively narrow and the general representation might be even lost, these simple patients with non-immunocompromised comorbidities were more able to reflect the expression changes of negative costimulatory molecules LAG3 and PD-1 in the case of sepsis, as well as the clinical prognosis changes brought by such changes. With this preliminary study, we can proceed to the related study of broad standard enrollment. As for the identification of sepsis on the 1st day, we made a daily diagnostic evaluation for the infected patients highly suspected for sepsis, using sepsis 3.0 criteria. In this study, we found that PD-1 and LAG3 have unique expression characteristics in sepsis. Although LAG3 plays an important role in T cell inhibition in other diseases, such as cancer (36–39), autoimmune diseases (40–42), chronic viral infections (43, 44), and parasites (13–15, 45), its role in sepsis is not well-understood. Given the changes in LAG3 and PD-1, we need to closely examine their specific roles in sepsis.

The function test confirmed that T cells with double-positive expression of LAG3 and PD-1 were significantly depleted, while T cells with single-positive expression of LAG3 or PD-1 still had certain secretory and proliferative capacities (**Figure 6**). Moreover, in most patients, the secretory function of the T cells with double-positive expression of LAG3 and PD-1 was twice as damaged as that of the T cells with any single-positive expression pattern. Similar results were also obtained by comparing the apoptosis rate and proliferative ability of the T cells with double-positive expression of LAG3 and PD-1 with those of any single-positive T cells (**Figures 7**, **8**). T cells with double-positive expression showed extremely weak proliferative ability, and more than 80% of the cells remained in the parental cell state (**Figure 8B**). Even if proliferation occurred, there were not many generations, with more cells in generation 3 or so; in contrast, the control group had more cells in the seventh generation and beyond. Likewise, T cells with single-positive expression of LAG3 or PD-1 still retain a certain proliferative capacity. There were many reports about the exhaustion of T cells caused by the increase of PD-1 expression (46–51), and a decrease in the function of T cells with single positive PD-1 expression also could be found in this acute sepsis study, but not significant exhausted. Maybe there are some other mechanisms that contribute to T

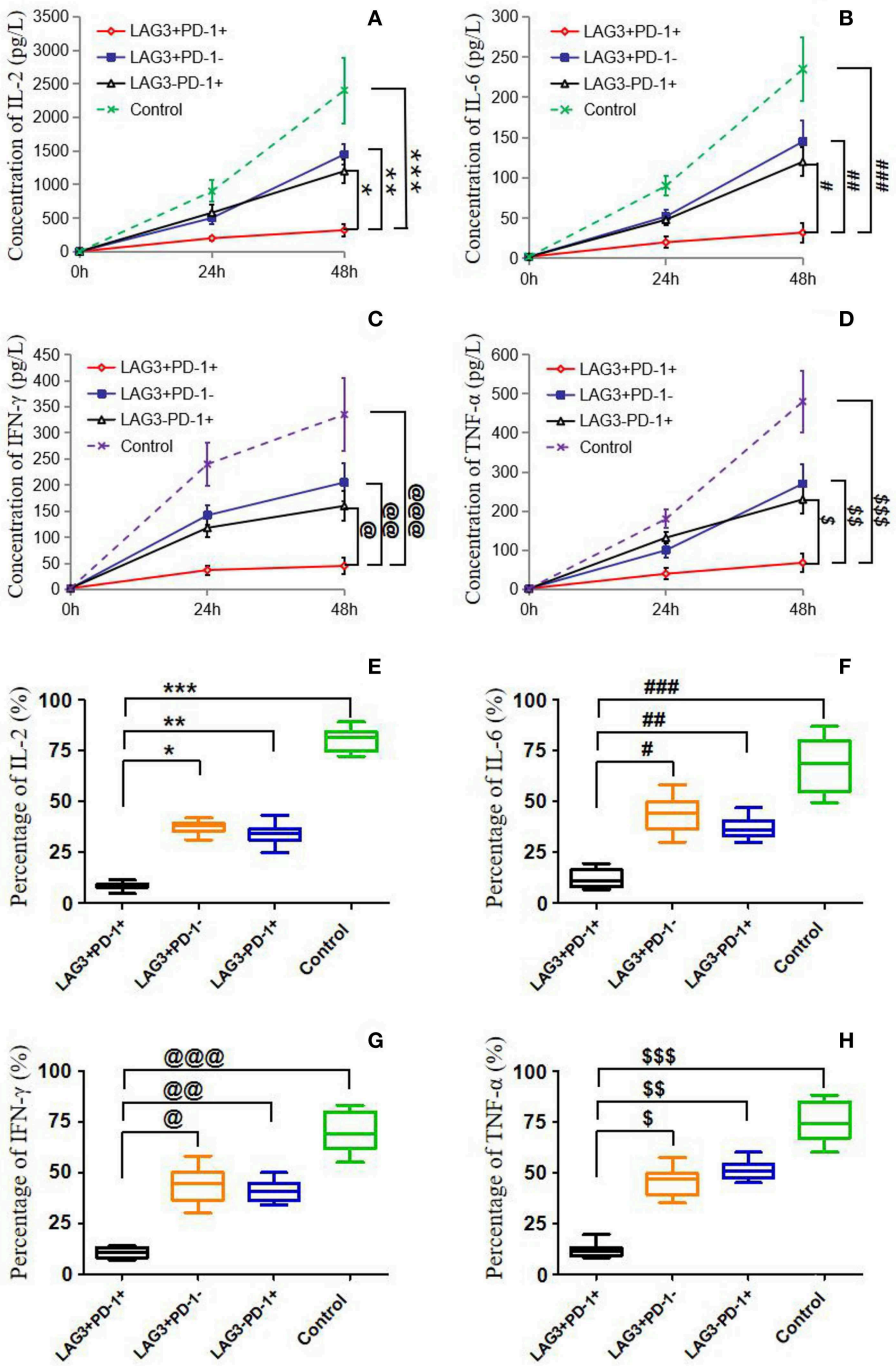

FIGURE 6 | Determination of the cytokine secretion by T cells with single- or double-positive expression of LAG3 and PD-1. As described before, T cells with different phenotypes were sorted via FACS, cultured in 24-well plates, activated by α-CD3/α-CD28 and stimulated with PMA (50 ng/ml) and ionomycin (500 ng/ml) for 48 h. Cytokine concentrations in the supernatant were determined by ELISA **(A–D)**, and intracellular cytokine levels were measured by flow cytometry **(E–H)**. IL-2 **(A,E)** and IL-6 **(B,F)** were measured mainly to evaluate the function of $CD4^+$ T cells, and IFN-γ **(C,G)**, and TNF-α **(D,H)** were measured mainly to detect the function of $CD8^+$ T cells. There was a common trend in cytokine secretion capacity, that is, the function of LAG3 and PD-1-coexpressing T cells was significantly weaker than that of LAG3 or PD-1 single-positive T cells and control T cells; $^*P < 0.05$, $^{**}P < 0.05$, $^{***}P < 0.05$; $^{@}P < 0.05$, $^{@@}P < 0.05$, $^{@@@}P < 0.05$; $^{\#}P < 0.05$, $^{\#\#\#}P < 0.05$, $^{\#\#\#}P < 0.05$; $^{\$}P < 0.05$, $^{\$\$}P < 0.05$, and $^{\$\$\$}P < 0.05$. In particular, the T cells coexpressing LAG3 and PD-1 exhibited decreased cytokine secretion that was more than 2-fold lower than the secretion of the single-positive T cells. Data of **(A–D)** are expressed as the mean ± SEM, and One-way ANOVA with Tukey's multiple comparison test was used. Data of **(E–H)** are shown as a box plot and analyzed using the Kruskal-Wallis Test due to variance inhomogeneity.

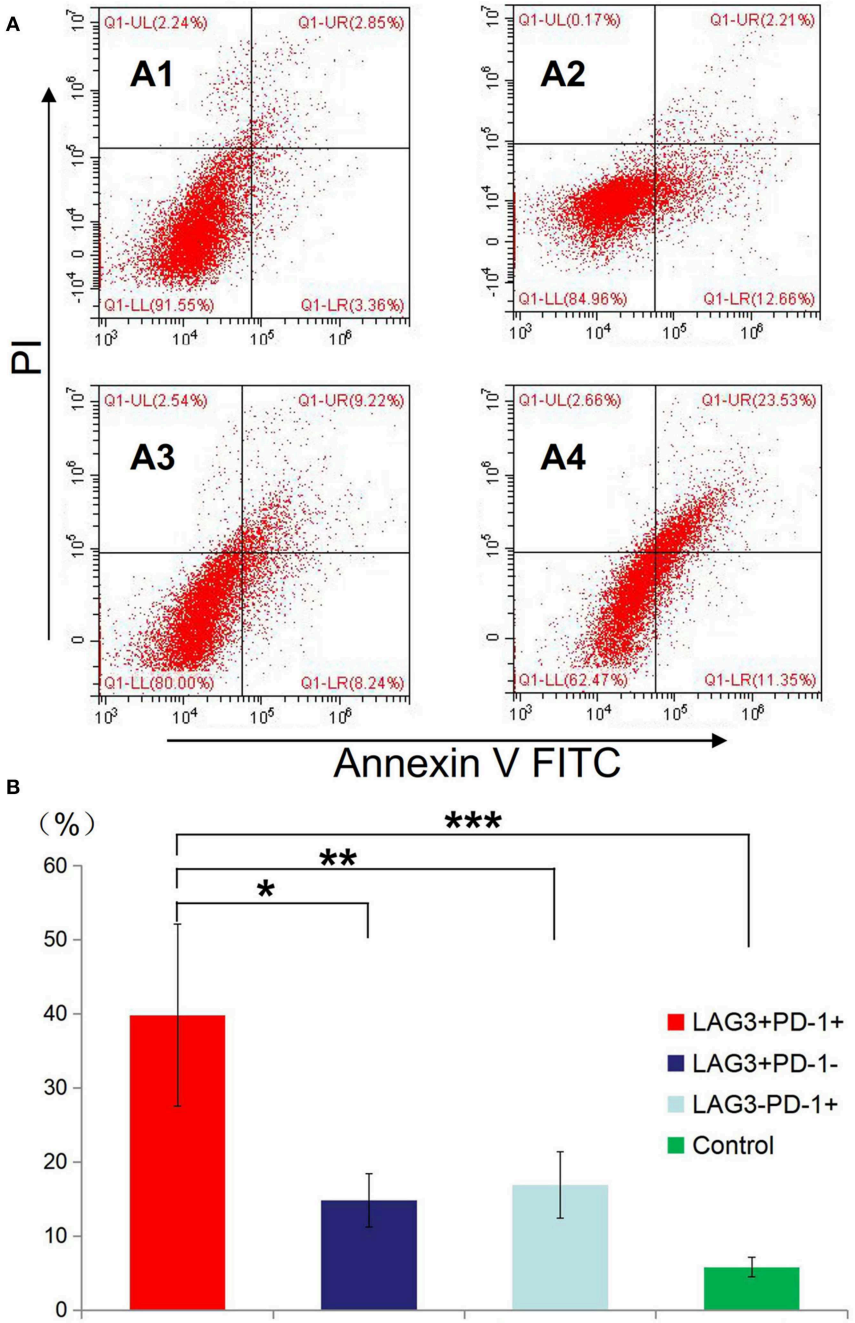

FIGURE 7 | Determination of the apoptosis rates of T cells with double- or single-positive expression of LAG3 and PD-1. T cells from the peripheral blood of subjects and controls were sorted and cultured for 48 h. The apoptosis rates of T cells with different phenotypes including the T cells from controls **(A1)**, $LAG3^{+}PD\text{-}1^{-}$ T cells **(A2)**, $LAG3^{-}PD\text{-}1^{+}$ T cells **(A3)**, and $LAG3^{+}PD\text{-}1^{+}$ T cells **(A4)** were determined by flow cytometry. We found that the apoptosis rates of the $LAG3^{+}PD\text{-}1^{+}$ T cells were significantly higher than those of the $LAG3^{+}PD\text{-}1^{-}$ T cells ($^{*}P < 0.05$), $LAG3^{-}PD\text{-}1^{+}$ T cells ($^{**}P < 0.05$), and control T cells ($^{***}P < 0.05$) **(B)**. The early and late apoptosis rates of the $LAG3^{+}PD\text{-}1^{+}$ T cells were far >2-fold higher than those of the $LAG3^{+}PD\text{-}1^{-}$ T cells and $LAG3^{-}PD\text{-}1^{+}$ T cells. Data are expressed as the mean ± SEM, and One-way ANOVA with Tukey's multiple comparison test was used.

cell exhaustion in chronic viral infections. In sepsis patients, the proportion of double-positive $CD8^{+}$ T cells was significantly higher than that of $CD4^{+}$ T cells, suggesting that the synergistic inhibitory effect of the two was more prominent on $CD8^{+}$ T cells, although the proportion of double-positive $CD4^{+}$ T cells was also significantly higher than that of the control group (**Figure 5**). Because sepsis is generally associated with an absolute decrease in the lymphocyte count, we further analyzed the relationship

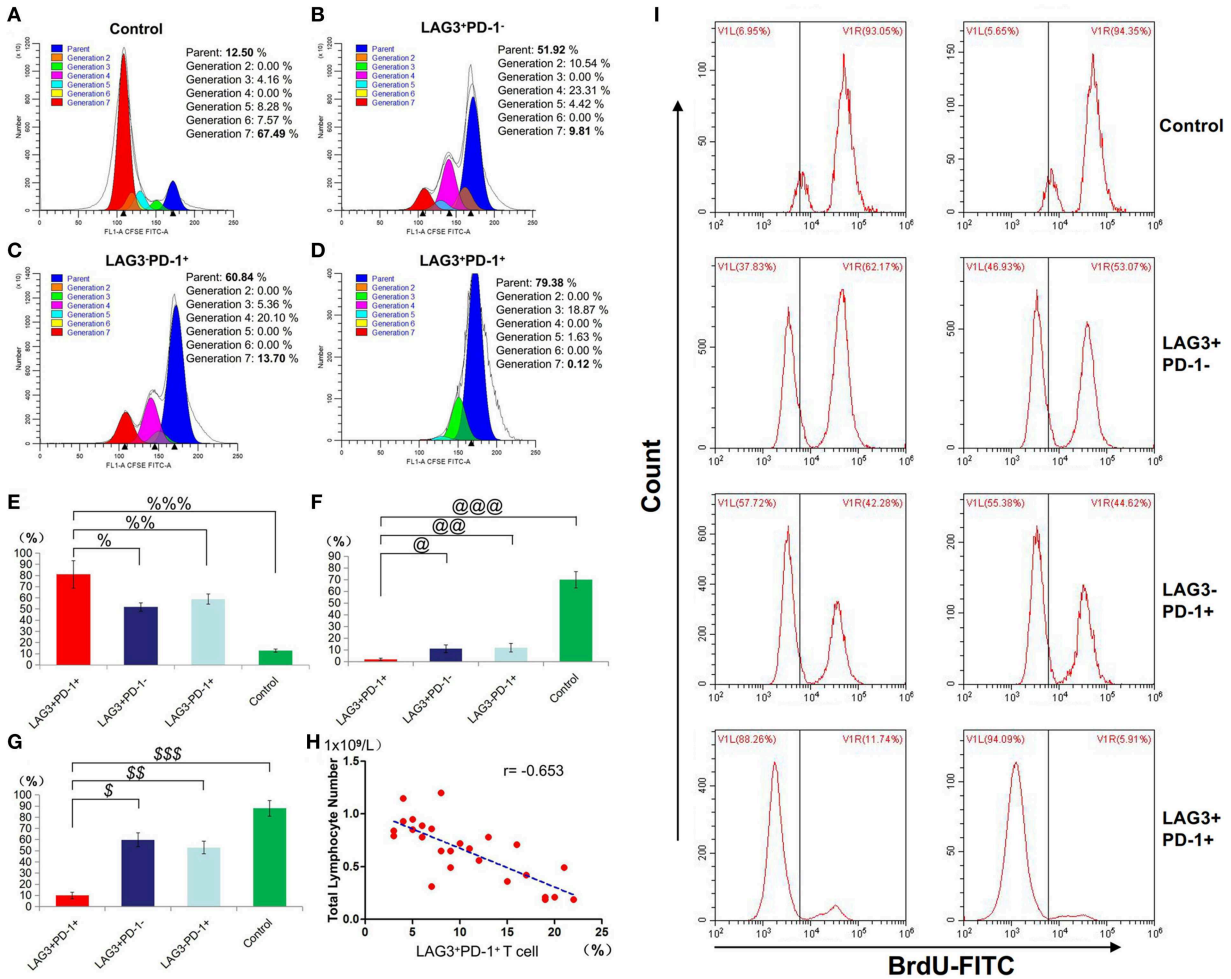

FIGURE 8 | Determination of the proliferation of T cells with double- or single-positive expression of LAG3 and PD-1. After the sorting and culturing described above, we used two methods, CFSE **(A–F)** and FITC-Brdu **(G)** staining, to consistently evaluate the proliferative capacities of T cells with different phenotypes. From the division and proportional analysis, we found that the proliferation of LAG3^{+}PD-1^{+} T cells was obviously inhibited **(A)** compared with that of LAG3^{+}PD-1^{-} T cells **(B)**, LAG3^{-}PD-1^{+} T cells **(C)**, and control T cells **(D)**, with the highest proportion of cells remaining in the parental generation ($^{\%}P < 0.05$, $^{\%\%}P < 0.05$, and $^{\%\%\%}P < 0.05$) **(E)** and the lowest proportion of cells appearing in the seventh generation ($^{@}P < 0.05$, $^{@@}P < 0.05$, and $^{@@@}P < 0.05$) **(F)**. The analysis of FITC-Brdu staining showed similar results, with the proliferation rates of LAG3 and PD-1 double-positive T cells being the lowest ($^{\$}P < 0.05$, $^{\$\$}P < 0.05$, and $^{\$\$\$}P < 0.05$) **(G)**. The proportion of coexpressing T cells was negatively correlated with the total number of lymphocytes using Pearson's simple correlation coefficient ($r = -0.653$, 95%CI: -0.831 to -0.356, $P = 0.0003$) **(H)**. Data of **(E–G)** are expressed as the mean ± SEM, and compared with ANOVA. Representative pictures of flow cytometry using BrdU-FITC to detect the proliferation rates of T cells with different phenotypes **(I)**.

between the proportion of T cells with double-positive expression of LAG3 and PD-1 and the absolute number of lymphocytes and found that there was a strong negative correlation between these parameters. This finding also confirmed the pathological nature of this relationship, as the proliferative capacity of LAG3 and PD-1 double-positive T cells was weakened while the apoptosis rate was increased. However, this relationship was not found between the absolute lymphocyte count and T cells with single-positive expression of PD-1 or LAG3 in this study.

Furthermore, we analyzed the relationships between the proportion of LAG3 and PD-1 double-positive T cells and relevant clinical indicators. Interestingly, the double-positive proportion was positively correlated with the degree of sequential organ injury (SOFA score) (**Figure 9A**). Further analysis showed that the higher the proportion of T cells with double-positive expression of LAG3 and PD-1 was, the more serious the organ damage, the longer the hospital stay, the higher the mortality, and the lower the survival rate (**Figure 9C**). In this study, the proportion of double-positive T cells was always below 25%, while the proportion of double-positive T cells in the control group was below 5%, and no septic patients died when the proportion of double-positive T cells was <5%. That is why we set 5% as a cut-off. Due to the potential synergistic effect of PD-1 and LAG3 and its significant influence on clinical prognostic indices, the therapeutic strategies for immunomodulatory therapy may need to be adjusted in the future. A previous study showed that

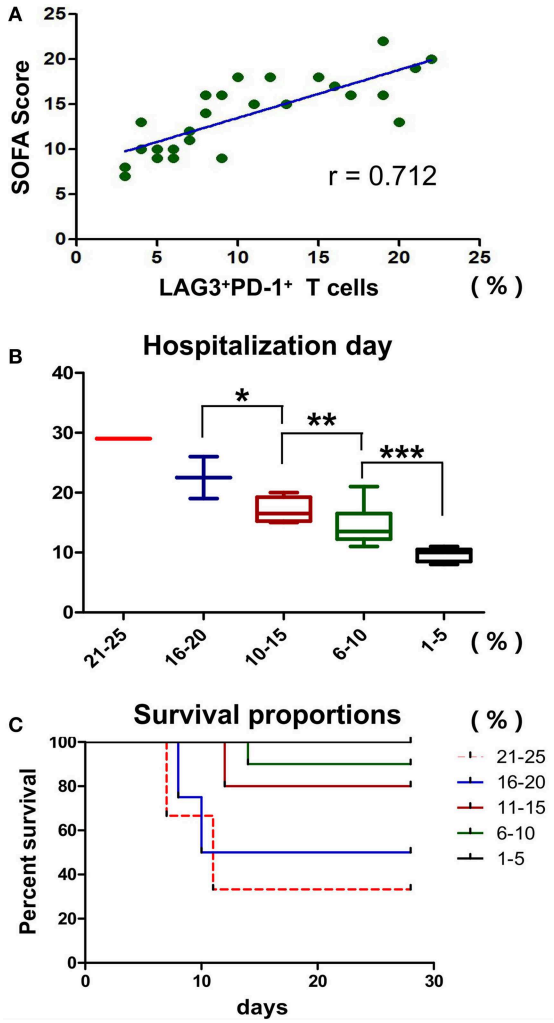

FIGURE 9 | Effects of T cells with LAG3 and PD-1 double-positive expression on the clinical indicators and prognosis of patients with sepsis. The proportion of coexpressing T cells was positively correlated with the SOFA score using Pearson's simple correlation coefficient ($r = 0.712$, 95%CI: 0.448 to 0.862, $P < 0.0001$) **(A)**. Due to the small number of patients, we compared the proportions of LAG3 and PD-1-coexpressing T cells by subgroups in the mortality and survival analyses, and we set 5% as the group increment. The numbers of patients with 1–5%, 6–10%, 11–15%, 16–20%, and 21–25% double-positive T cells were 5, 9, 5, 4 and 3, respectively. The higher the proportion of coexpressing T cells was, the longer the hospital stay (*$P < 0.05$, **$P < 0.05$, and ***$P < 0.05$) **(B)**, analyzed by Kruskal-Wallis Test. For the survival analysis, a log-rank test was performed. We found that there were differences in survival among the high-proportion subgroups ($P < 0.05$, a vs. b; $P < 0.05$, a vs. c; $P < 0.05$, b vs. c; $P < 0.05$, a vs. d; and $P < 0.05$, a vs. e) and no differences among the low-proportion subgroups ($P > 0.05$, e vs. d and $P > 0.05$, d vs. c) **(C)**.

delaying the use of PD-1 blockade to after 24 h of sepsis could improve the survival of mice with sepsis to some extent (52). When that result is combined with our finding of the unique expression features of PD-1, it seems that the expression of PD-1 in acute sepsis is more likely to be passively increased to prevent the uncontrolled inflammatory cascade and the late coexpression of LAG3 may be the key to T cell exhaustion. Therefore, the use of anti-PD-1 treatment in too early or too late periods will not produce satisfactory therapeutic effects. When used too early, the cascade of inflammatory responses can get out of control, resulting in more early death. When used in too late stages, the activation of LAG3 will certainly affect the effect of the anti-PD-1 therapy, not only in sepsis but also in cancer (16).

Although we systematically analyzed the expression characteristics and functional relationship between LAG3 and PD-1 in T cells from septic patients, and obtained some important results, which may lead to a change in the strategy of immunomodulatory therapy for sepsis in the future, there is still a small limitation. Due to the strict exclusion criteria, although we had many candidates, the number of patients actually included in the study was small. In addition, although we revealed a potential synergistic role for LAG3 and PD-1 in mediating the progressive depletion of T cells, this study, like other studies (12, 18, 19, 53), was not been able to elucidate the mechanism of this synergistic effect because the downstream signaling pathway of LAG3 is poorly understood at present (13). However, we believe that future researchers will be able to shed light on the synergistic mechanisms between LAG3 and PD-1, and multicenter, larger sample clinical studies are expected to confirm the significance of this synergy to help improve the clinical management and prognosis of patients as early as possible.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

This study was approved by our local ethical review committee in compliance with the declaration of Helsinki. Written and informed consent was obtained from all patients enrolled. (Ethics Committee of the First Affiliated Hospital of Chongqing Medical University, Chongqing, China, the ethical document NO. 2018-017).

AUTHOR CONTRIBUTIONS

BN and GR designed the experiments. BN, HD, YS, YX, and ZY performed the experiments and analyzed the data. FZ, YS, LW, and YW collected and analyzed the clinical data. GR further polished the manuscript. GR and HD authorized the publication of the manuscript.

FUNDING

This work was supported by the Fostering Foundation of the First Affiliated Hospital of Chongqing Medical University (PYJJ2017-26), the Yuzhong District Science and Technology Project (20170408), the Intensive Care Medical Research Fund of AESCULAP (2017001), and the Scientific Research Fund of Chongqing Medical University (NSFYY201705).

ACKNOWLEDGMENTS

This study was performed by the Chongqing Key Laboratory of Molecular Oncology and Epigenetics, the Department of Emergency and the Department of Critical Care Medicine of the First Affiliated Hospital of Chongqing Medical University. We are thankful for the help of Dr. Weiyan Peng, Dr. Zhu Qiu, Dr. Jun Tang, Qian Wu, Chan Bi, Ji Tang, and Xueyuan Wang in the Chongqing Key Laboratory of Molecular Oncology and Epigenetics.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 25 September 2019
doi: 10.3389/fimmu.2019.02247

Immunosenescence and Its Hallmarks: How to Oppose Aging Strategically? A Review of Potential Options for Therapeutic Intervention

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OPEN ACCESS

Edited by:
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James Edward Turner, University of Bath, United Kingdom

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Specialty section:
This article was submitted to Immunological Memory, a section of the journal Frontiers in Immunology

Received: *29 March 2019*
Accepted: *05 September 2019*
Published: *25 September 2019*

Citation:
Aiello A, Farzaneh F, Candore G, Caruso C, Davinelli S, Gambino CM, Ligotti ME, Zareian N and Accardi G (2019) Immunosenescence and Its Hallmarks: How to Oppose Aging Strategically? A Review of Potential Options for Therapeutic Intervention. Front. Immunol. 10:2247. doi: 10.3389/fimmu.2019.02247

Aging is accompanied by remodeling of the immune system. With time, this leads to a decline in immune efficacy, resulting in increased vulnerability to infectious diseases, diminished responses to vaccination, and a susceptibility to age-related inflammatory diseases. An age-associated immune alteration, extensively reported in previous studies, is the reduction in the number of peripheral blood naïve cells, with a relative increase in the frequency of memory cells. These two alterations, together with inflamm-aging, are considered the hallmarks of immunosenescence. Because aging is a plastic process, it is influenced by both nutritional and pharmacological interventions. Therefore, the role of nutrition and of immunomodulation in immunosenescence is discussed, due to the multifactorial influence on these hallmarks. The close connection between nutrition, intake of bioactive nutrients and supplements, immune function, and inflammation demonstrate the key role of dietary strategies as regulators of immune response and inflammatory status, hence as possible modulators of the rate of immunosenescence. In addition, potential options for therapeutic intervention are clarified. In particular, the use of interleukin-7 as growth factor for naïve T cells, the function of checkpoint inhibitors in improving T cell responses during aging and, the potential of drugs that inhibit mitogen-activated protein kinases and their interaction with nutrient signaling pathways are discussed. Finally, it is suggested that the inclusion of appropriate combinations of toll-like receptor agonists may enhance the efficacy of vaccination in older adults.

Keywords: aging, immunosenescence, immunomodulation, immunotherapy, nutrition

INTRODUCTION

People worldwide are living longer. In 2025, there will be about 1.2 billion people over the age of 60, increasing to 2 billion by 2050 (1). However, the increase in lifespan does not coincide with the increase in healthspan, i.e., the period of life free from serious chronic diseases and disability. In fact, the influence of aging on humans is responsible for physiological dysfunctions

in the different tissues, organs, and systems, including the immune system (2, 3). The age-related involvement of immune system leads to a progressive reduction in the ability to trigger effective antibody and cellular responses against infections and vaccinations. This phenomenon, called immunosenescence, a term coined by Roy Walford, is multifactorial, and affects both natural and acquired immunity, although T lymphocytes are dramatically affected (4). In fact, aging process more extensively affects acquired immunity than innate immunity (3, 5). Several factors, such as genetics, nutrition, exercise, previous exposure to microorganisms, biological and cultural sex, and human cytomegalovirus (HCMV) status can influence immunosenescence (3, 6–11).

Concerning sex, steroid hormones, linking to specific receptors, differentially modulate the immune system. In general, while estrogens increase the immune response, progesterone and androgens have immune suppressive actions. However, a few studies have analyzed the post-menopausal immune system (12). Therefore, it is unclear whether age-related changes in the immune system are different between men and women, although some data show that immunosenescence develops earlier in men than in women. This has been related to longer life expectancy of women (8, 13, 14). In addition, no evidence exists that males and females respond differently to therapeutic intervention against immunosenescence.

Many studies have emphasized the importance of viruses, such as herpes viruses, responsible for both latent and chronic infections, in shaping T cell compartments during aging (15). In particular, HCMV seropositivity seems related to many functional T cell changes. HCMV status has a greater impact than age on the immune system, because the virus contributes to shape the immune profile and function during normal human aging (16–18).

Understanding mechanisms of age-related disorders in immune regulation is important to identify more efficient strategies for immune rejuvenation and for effective induction of vaccination-mediated immunity in older individuals. Aging is a malleable process, affected by both nutritional and pharmacological interventions (19, 20). Therefore, immune system might also be prone to intervention. However, all possible therapies aimed at non-specifically "rejuvenating" the immune system might be counterproductive. In fact, the different parameters observed between young and older people could also be a product of the adaption vs. the exposome, i.e., all the stimuli that the immune system has undergone during life. Therefore, a targeted intervention for safe "rejuvenation" of the immune status in older people should be necessary (21).

Within the past years, numerous studies of underlying mechanisms of age-related immune decline have laid the groundwork for the identification of targeted approaches (5, 22–24). We will discuss below the most relevant strategies currently being investigated. We will also considered the role of nutrition in immunosenescence and in its counteraction in the section on dietary strategies currently being investigated. Further, we will examine the available data on growth factors [i.e., on interleukin (IL)-7], on monoclonal antibodies (MoAbs) that affect immune checkpoints, and on drugs that inhibit mitogen-activated protein kinases (MAPK) and their interaction with nutrient signaling pathways. These treatments, representing a promising therapeutic approach, will be treated in the section on clinical approaches. In the section on the other approaches in development, we will suggest that the inclusion of appropriate combinations of toll-like receptor (TLRs) agonists might enhance the efficacy of vaccination-mediated immunity in older adults. Finally, at the end of conclusion, we will outline possible future approaches.

SUMMARY OF IMMUNOSENESCENCE

Innate Immunity

The general picture of innate immunity in older people, which emerges from several studies, is that of the down-regulation of some functions and the up-regulation of others. We will discuss data on dendritic cells (DCs) due to their relevance for the immunotherapeutic approaches, including vaccination. For the other aspects of innate immunity in older individuals, see (3, 25, 26). Briefly, natural killer (NK) cell cytotoxicity is well-preserved in centenarians, and an increase in the actual number of NK cells is observed in healthy aging. Neutrophils show reduced function in bacteria phagocytosis and in the oxidative burst while macrophages show reduced chemotaxis and phagocytosis, and decreased cytokine production.

DCs, the most potent antigen presenting cells (APCs), can be divided into three subsets according to the expression of various markers (CD123, CD1c, CD141), one subset of plasmacytoid DCs (pDCs) and two subsets of myeloid DCs (mDCs) (27). Both pDCs and mDCs express TLRs that recognize conserved pathogen-associated molecular patterns (PAMPs) on microbes, and are key regulators of antimicrobial host defense responses. The type of TLR-activated DC determines the cytokine pattern (28).

There are discordant data on age-related changes in the frequency and absolute number of pDCs and mDCs. Regarding the ability to secrete cytokines upon stimulation, there are apparent inconsistencies in the available data for mDCs from older population. pDCs are instead characterized by a marked impairment of cytokine release in older people (27, 29). Recognition of microbial components by TLRs culminates in the secretion of type I interferons (IFNs) and cytokines that facilitate the coordination of innate to acquired immune responses. Peripheral blood mononuclear cells (PBMCs) isolated from older individuals ($\geq$65 years) exhibited a delayed and altered response to stimulation with TLR agonists compared with cells obtained from young adults ($\leq$40 years). This delayed response to agonists results in the reduced production of cytokines and chemokines (29). On the other hand, the addition of PAMPs to a subunit vaccine, triggering their corresponding pattern-recognition receptors (e.g., TLRs) improves vaccine efficacy in older humans and mice (25, 30–32). Accordingly, DCs together with naïve T cells represent the most restrictive elements for the immune response to primary viral infections in older people (33).

As the expression of TLRs remains constant during life, defects in signal transduction should be responsible for this impairment, as discussed by (24).

Acquired Immunity and the Hallmarks of Immunosenescence

The quality and quantity of the T and B cell responses change with increasing age, with consequent changes on the effectiveness of the immune response. This leads to an inadequate immune response against newly encountered antigens. The apparently inevitable consequence of this complex scenario is the reduced ability of older individuals to respond to novel antigens and to vaccines, resulting in an increased susceptibility to infection and in the development of age-related diseases, including cancer (3). As critically reviewed by (3, 6, 16), a number of longitudinal studies of octogenarians and non-agenarians performed in Sweden defined an immunological risk phenotype (IRP). Participants with the reversal of CD4/CD 8 T-cell ratio, a reduced proliferative response to mitogenic stimuli, and severe reduced B cells number showed reduced survival. Subsequently, the data were implemented and related to HCMV seropositivity, because HCMV seropositivity is closely related to the reversal of CD4/CD 8 T-cell ratio. In fact, as discussed below, persistent HCMV infection leads to chronic stimulation of CD8 T cells, which expand clonally showing an effector memory phenotype characterized by low CD28 expression. The IRP was present in around 15% of 85-years-olds in these studies at baseline. Follow-up of 2-, 4-, and 6-years mortality revealed significantly higher all-cause mortality in the IRP group than in the majority of other octogenarians and non-agenarians. However, this IRP was not confirmed in the Leiden 85-Plus study, a prospective population-based cohort study of individuals at the age of 85 years living in Leiden (NL). Thus, immune parameters associated with survival may vary in diverse populations at different ages (6). Therefore, we focus on the changes we have considered the hallmarks of immunosenescence, based on the literature data (6, 23).

The hallmarks of immunosenescence include: (i) a reduced ability to respond to new antigens; (ii) the accumulation of memory T cells; (iii) a lingering level of low-grade inflammation termed "inflamm-aging." Mechanistically, immunosenescence is only partially explained by organismal and cellular senescence. Therefore, these hallmarks of immunosenescence would be markedly affected by the history of the individual exposure to pathogens (6, 23).

The reduced ability to respond to new antigens is linked to a decreased number of peripheral naïve T and B cells (see last paragraph of this section). Naïve T cells are abundant in youth but may become "used up" by exposures to microorganisms over the course of life, hence differentiating into memory lymphocyte subsets. In addition, their number decreases following the involution of primary lymphoid organs, because age-related defects have been observed in their stroma. Some changes occur early in the developmental progression from hematopoietic stem cells (3). Thymus involution occurs at the time of puberty, and is characterized by atrophy and replacement by adipose tissue. This process seems related to the increase of sex hormones and to the decrease of IL-7, a hematopoietic growth factor secreted by stromal cells in the bone marrow and thymus. IL-7 exerts its action through the binding to a heterodimeric receptor composed of an α chain (IL-7Rα or CD127) and the common cytokine receptor γ chain (γc or CD132). CD127 is expressed on lymphoid lineage cells at different stages of development, whereas CD132 is shared with other cytokine receptors and expressed on most hematopoietic cells (34, 35). Irrespective of thymic activity, the naïve compartment only moderately decreases in size during the following life decades, while mostly maintaining overall diversity and distribution of clonal sizes. An abrupt contraction is seen in later life. Therefore, at the age of 50, T cell production is <10% of its previous peak levels. From an evolutionary point of view, this occurs because exposure to new pathogens is maximal during the first years of life, but less likely in later life when immune memory for previously encountered pathogens is both more prevalent and more significantly important for survival (3, 22, 36).

The life-long chronic antigen load causes the filling of the immunological space by a population of T lymphocytes with a late-differentiated phenotype and the shrinkage of the T cell repertoire. As previously stated, an age-related decrease in absolute number of peripheral blood naïve T cells is consistently found in all studies and in different human populations (22, 37). Due to the lifelong and chronic exposure to pathogens, T cells replicate several times and become late-differentiated effector memory T cells with features of replicative senescence (38). T cell senescence focuses on the phenotypic characteristics of individual lymphocytes and refers mainly to a low proliferative activity (39). Aging *per se* leads only to a relative accumulation of memory cell subsets, linked to the decrease in naïve cell populations. The absolute increase in memory T cells, called memory inflation, is observed only in older people infected by HCMV (40). These T cells do not express the co-stimulatory molecule CD28, required for the activation of T cells. The loss of CD28 occurs following cell proliferation, according to the observation that the $CD28^-$ T cells have shorter telomeres than $CD28^+$ cells. These CD28− cells express high levels of the adhesion molecule integrin CD11a/CD18 and have high levels of perforin and granzyme, responsible for the killing of the target cells. CD28 seems a good biomarker of immunosenescence, as further suggested by findings that late-differentiated $CD8^+/CD28^-$ T cells tend to accumulate particularly in older people, frail or affected by age-related diseases. These cells display a highly differentiated phenotype, expressing CD27, another co-stimulatory molecule, but not CD28 (however, in $CD28^+$ subset, $CD28^-CD27^-$ seem to be more frequent). They also carry short telomeres, lack telomerase and express negative signaling receptors, such as programmed cell death protein (PD)-1, which is involved in the down-regulation of the immune system (see paragraph on checkpoints inhibitors; the example of PD-1 and CTLA-4). Senescent T cells also express CD57 displaying a high cytotoxic potential, and killer cell lectin-like receptor subfamily G member 1. Late-stage memory senescent T-cells may also acquire new functions, such as suppressive activity, as demonstrated *in vitro*. In addition, they are producers of pro-inflammatory cytokines (17, 18, 41–47). However, a longitudinal study of 249 research participants followed for 10 years has strongly suggested that HCMV infection is not a primary causative factor in the age-related increase in systemic inflammation (48). Therefore, the accumulation of

memory T cells, especially late-stage differentiated $CD8^+$ cells is viewed as the result of depletion of the reservoir of naïve cells over time by contact with pathogens and their conversion to memory cells. However, the memory responses can be unsustained, because T cell memory established in humans during early age can deteriorate during the second half of life. The most obvious example of unsustained memory responses is the reactivation of latent varicella zoster virus (VZV) infection that manifests as herpes zoster. A steady decline of VZV-specific $CD4^+$ T cells over time has been documented, which is only very transiently boosted with zoster vaccination or reactivation (49). In contrast, high frequencies of antigen-specific T cells reactive to HCMV persist throughout life. T cell clones specific for HCMV dominate the repertoire in the older people and contribute to the contraction in diversity in the memory compartment (23).

Nearly 20 years ago, Looney et al. reported the dramatic impact of HCMV on the immune system of older people (50). This observation was subsequently described in numerous other studies (18, 46). In the latent state, the intermittent production of viral antigens prevents contraction of virus-specific T cells. Therefore, the virus is responsible for the generation of a large population of HCMV-specific $CD8^+$ T cells, with a significant increase in highly differentiated $CD8^+$ effector memory T cells, which expand clonally showing an effector memory phenotype characterized by low CD28 expression. As previously stated, this determines the phenomenon of memory cell inflation, leading to the emergence of vast populations of resting effector $CD8^+$ and, to a lesser extent, $CD4^+$ cells. In older people, one or a few clonal populations can occupy more than 25% of the entire $CD8^+$ cell pool (46, 51). These inflated HCMV-specific memory T cells maintain their efficient effector functions for the lifetime of the individual (40, 46, 52). Inflationary $CD8^+$ cells, after proper activation stimuli, can divide, secrete cytokines, and execute cytolysis, i.e., they are not exhausted. However, there may be a slight loss of control of HCMV replication in older compared with younger people. In fact, HCMV load in blood markedly increases in healthy people over the age of 70 years (53). Immune changes associated with HCMV may have significant impact during co-infection and vaccination, as well as on general and immunological fitness. However, the correlation between HCMV positivity and impaired responses is controversial because this relationship is observed in some but not all studies (54–56).

Persistent antigenic challenges lead to a poor response to newly encountered microbial antigens, as well as to a shift in the immune system toward an inflammatory, autoimmune, T helper (Th) 2 profile. In addition, the long-term chronic microbial burden induces progressive activation of macrophages, hence contributing to the chronic state of low-grade inflammation, inflamm-aging, another hallmark of immunosenescence (3, 9, 57). This term defines the systemic state of chronic low-grade inflammation considered a central biological pillar of the aging process and a common pathogenetic mechanism of age-related diseases, as well as a worse prognostic factor for all causes of death (9, 57–59). In the course of aging, there is a reduction in the ability to endure consequences of antigenic, chemical, physical, and nutritional triggers of inflammation. Chronic and low-grade inflammation can lead to tissue dysfunction and degeneration. Our immune system is quite efficient in fighting acute infections in young people, but not particularly efficient in responding to chronic stimuli, especially when they occur late in life. This leads to an increased production of pro-inflammatory cytokines and acute phase proteins (59, 60). Oxidative stress also plays an important role in determining and maintaining this low-grade inflammation, which, in turn contributes to oxidative stress (61, 62). Inflamm-aging results from the activation of signaling networks critical to inflammation, such as those regulated by the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transcription factor, particularly when combined with a variety of stimuli, such as senescent cells, obesity, circulating mitochondrial DNA, gut microbiota and diet triggering and sustaining inflammatory conditions (58, 63–68). However, as previously stated, immunosenescence represents the most important contributor to inflamm-aging, in turn, contributing to impaired immune responses. In fact, inflamm-aging is responsible for a high expression of micro (mi)-RNAs that interfere with B cell activation, driving tumor necrosis factor (TNF)-α production and inhibiting B cell activation as measured *in vitro* (69). Increased serum levels of TNF-α are also linked to a defective T cell response, in part due to reduced expression of CD28 (21). Accordingly, in monocytes, the pre-vaccination expression of genes related to inflammation and innate immune response is negatively correlated to vaccination-induced activation of influenza-specific antibody responses (70).

Age-related B cell changes are similar to those observed in T cell compartment and the effects on humoral immune response are detrimental as well. Age also affects B cell numbers and B cell repertoire diversity, as well as immunoglobulin isotypes and receptor repertoire with a decrease in specific humoral immune responses against new extracellular pathogens (71). Activated B cells isolated from older adults display a reduced induction of E47, a class I basic helix-loop-helix protein encoded by the E2A gene. This is the key transcription factor, for the induction of activation-induced cytidine deaminase (AID), involved in class switching and somatic hypermutation. The reduced expression of E2A might be responsible for the decreased avidity of antibodies and diminished antibody-mediated protection (72, 73). This defect might be linked to a reduced interaction with $CD40L^+$ T helper cells, because, in older adults, the memory/effector T cells show a reduced expression of CD40L, necessary for B cells cooperation (74). The reduced levels of E47 and AID mRNA in B cells from older individuals are also due to the reduced mRNA stability. It is due to the higher expression of the inflammatory mi-RNAs 16 and 155, which bind to the 3'-untranslated region of E47 and AID mRNA, respectively, inducing mRNA degradation (69). In addition to the decrease in circulating B lymphocytes, there is a shift from immunoglobulin produced by naïve cells (IgD, IgM) to immunoglobulin produced by memory B cells (IgG, IgA). This is accompanied by an impaired ability to produce high affinity protective antibodies against infectious agents and the shrinkage of the repertoire diversity. The reduced serum levels of IgM and IgD suggest a shift in the balance from the naïve (CD27) toward the memory compartment ($CD27^+$), although this is not observed in all studies (71, 75–77).

See **Figure 1** for the schematic changes occurring during aging.

DIETARY STRATEGIES CURRENTLY BEING INVESTIGATED

There is a mutual interaction between nutrition, intake of particular bioactive dietary components, immune function, and inflammatory status, hence a close relation with the hallmarks of immunosenescence (78, 79). Many existing data demonstrate the key role of foods as regulators of immune response and inflammatory status, hence as possible modulators of the rate of immunosenescence, particularly inflamm-aging (11, 80). Other data have demonstrated the importance of following a specific, even personally tailored, dietary pattern (81). However, the intricate cellular and molecular network of immune system makes difficult to identify targeted strategies to rejuvenate specific compartment of immunity. Starting from supplementation with a single nutrient, leading to the application of experimental dietary pattern, much progress has been made in this field. The main barrier to better clarity remains the wide heterogeneity among human beings, linked to different life-style and genetic factors that influence the rate of immunosenescence (6, 10, 11, 82). Data discussed below show that the main target of dietary strategies is inflamm-aging, because diet, biotics, and nutraceuticals can show anti-inflammatory and anti-oxidant properties.

Diet

The high rate of long living people and the low incidence of cardiovascular disease in many Mediterranean countries suggest the importance of a diet rich in fruits, vegetables, whole grains, legumes, and olive oil (probably the main anti-aging food in this area). The reduced consumption of animal proteins, in particular red and cured meat, is also important. The efficacy of this diet results as an attenuation of inflammation and oxidative stress, and from the maintenance of a condition of eubiosis of the microbiota, involved in the general improvement of immune response in these populations (68, 83–86). In particular, the Mediterranean diet down-regulates the levels of inflammatory mediators, such as soluble intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM-1),

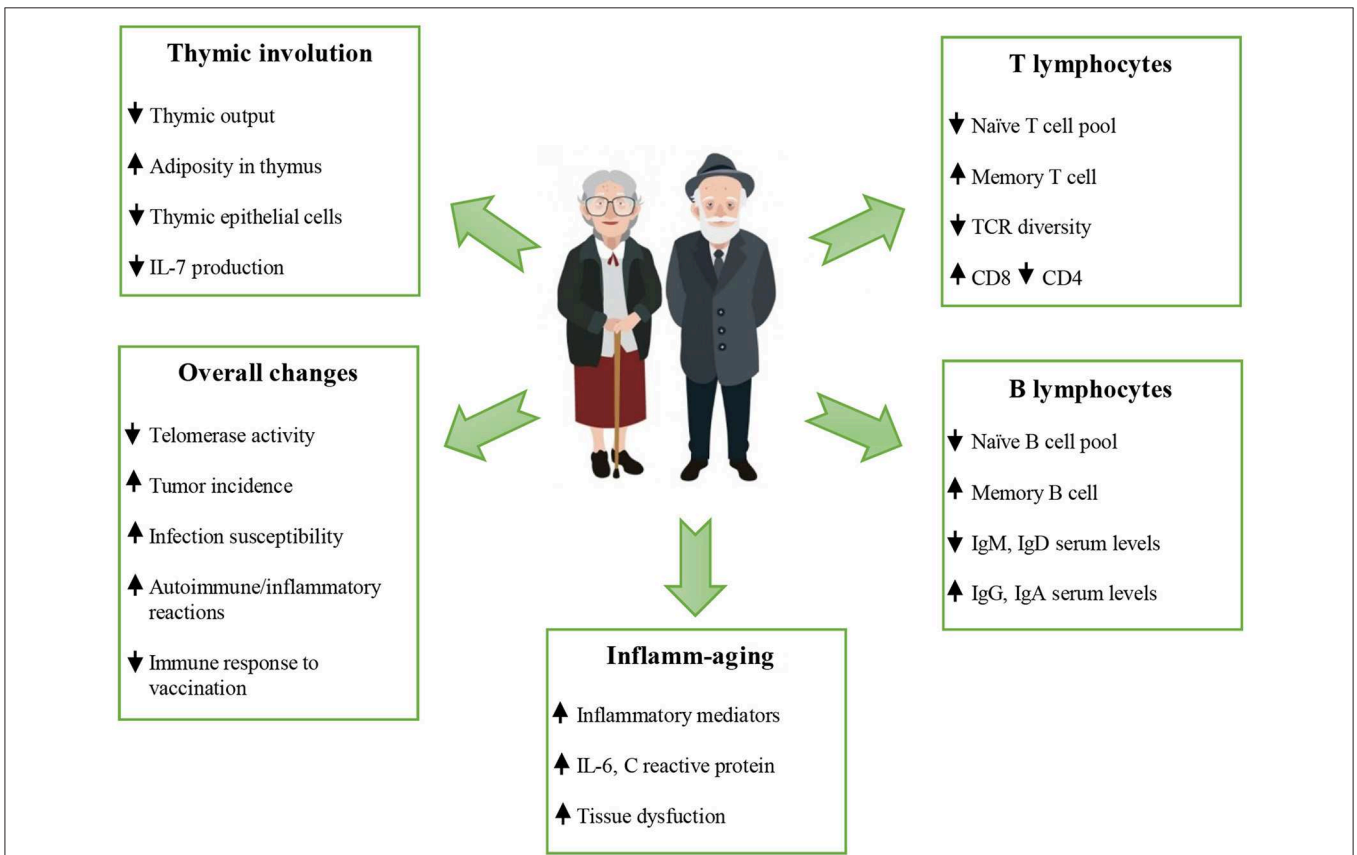

FIGURE 1 | Schematic changes occur during aging. The immunosenescence in older people is characterized by thymic involution, altered T and B cell responses, altered naïve/memory *ratio,* increased serum levels of IgG and IgA, a chronic low grade inflammation, and a poor response to newly encountered microbial antigens, including vaccines. The reduced ability to respond to new antigens, the accumulation of memory T cells and the lingering level of low-grade inflammation "inflamm-aging") are considered hallmarks of immunosenescence. See the text for the acronyms. ↑ increase; ↓ decrease.

C reactive protein, and IL-6, as well as many other biomarkers of inflammation (87–90).

A new interesting approach related to the possible reversion of immunosenescence is caloric restriction. NF-κB, mechanistic target of rapamycin (mTOR), and MAPK, pathways closely related to aging and inflammation are modulated by caloric restriction that downregulates the activation of IL-1β, IL-6, and tumor necrosis factor (TNF)-α genes, hence the pro-inflammatory state (20, 91, 92). More specifically, the results obtained by administration of different cycles of fasting, mimicking diet or long-term fasting influences inflamm-aging (20). These dietary patterns explicate their activity, particularly, during the refeeding period, reducing the rate of aging because of their antioxidant and anti-inflammatory effects, and possibly counteracting some other aspects of immunosenescence. The hypothetical explanation might be the disposal of damaged cells with growth of new functional cells.

Minor effects on immune function, after a brief starvation period of 72 hours, were seen in ten healthy, normal-weight, young volunteers. They showed an increase in suppressor cell numbers but no change in the number of peripheral blood leucocytes or in the differential counts (93). Unfortunately, these studies are severely limited by their complexity, further confounded by the small number of cases analyzed and the poor participants compliance (81).

New insights may also come from the use of caloric restriction mimetics, such as metformin, an activator of 5′ AMP-activated protein kinase (AMPK). It is a drug, typically administrated in type 2 diabetes but proposed as an anti-aging molecule for humans, such as the study called "Targeting Aging with Metformin" (94). Metformin can trigger AMPK, a pathway activated by energy depletion, i.e., by low levels of intracellular adenosine triphosphate (ATP), leading to the extension of healthy lifespan in model organisms (95). In mice with collagen-induced arthritis, metformin administration had an anti-inflammatory effect on arthritis due to the inhibition of Th17 cell differentiation, a subset of pro-inflammatory cells producing IL-17, and the upregulation of T regulatory cells (Tregs) differentiation along with the suppression of osteoclast differentiation (96, 97). Contrarily to caloric restriction, undernutrition, which is common in older people, is associated with an immunocompromised state, linked to altered T cell numbers, a reduced response to antigens, impaired release ofmediators, such as cytokines, and decreased phagocytosis and NK cell activity. This makes older people enable to trigger an efficient immune response to newly encountered pathogens. In such conditions of poor nutrition, the use of supplements, such as zinc, copper, iron, vitamins, nutraceuticals, and probiotics could be desirable and more appropriate than caloric restriction, as demonstrated by previous studies (98, 99).

Micronutrients

Nutritional status is crucial for the health status of older adults. Changes in phenotypic features, mainly loss of teeth and alterations in taste receptors, and gut disorders as well, determine a variation in both quality and quantity of food intake, contributing to general alterations in metabolism (100). Many studies have examined the influence of micronutrients and their influence on the enhancement of immune function in older adults (11, 79, 101, 102). Micronutrients, such as vitamins and minerals, are essential for the efficient performance of the immune system. They are needed in trace quantities, because the homeodynamic range is small, but the maintenance of a correct amount and balance is very rare in older people (often even in adults and young), both for scarcity and for excess due to unnecessary supplementation (78, 103).

One of the main micronutrients related to physiologic processes associated with immune system, and one of the main studied factors, is zinc. It is involved in many molecular processes, such as signal transduction, apoptosis, proliferation, and differentiation of cellular components of the immune system. Even slight deficiencies in zinc can have important consequences (104–107). Zinc deficiency can cause decreased levels of serum thymulin, a zinc dependent peptide hormone produced by thymic epithelial cells, with an activity that is progressively reduced with age, with a peak in pre-adolescence (78, 104, 108). The active form of thymulin induces the expression of markers of T lymphocyte activation, promoting T-mediated functions, acting both on the early and on the late phases of lymphocyte differentiation (109, 110). As shown in a randomized, doubleblind, placebo-controlled trial, after zinc supplementation for 12 months (45 mg elemental Zn gluconate/day), the incidence of infections was significantly lower, plasma zinc was significantly higher, and generation of TNF-α and oxidative stress markers was significantly lower in the zinc-supplemented participants than in the placebo group (both groups composed of 55–87 years old persons). Another doubleblind, randomized, controlled trial performed with zinc supplementation in old people (25 mg as zinc sulfate, once a day for 3 months, mean age of placebo group 80.6 ± 7.8, mean age of supplemented participants 79.5 ± 6.8) demonstrated increased levels of activated T helper and cytotoxic T lymphocytes, with a higher relative percentage of T cells with respect to the total circulating lymphocytes in zinc-supplemented older adults (105, 106, 111). Given the dose-dependent effect of zinc, both as a pro- and anti-oxidant, its presence in the normal range is essential for regulating the levels of reactive oxygen species (112). These studies highlight the importance of the zinc for immune function, but contrasting results exist, possibly reflecting the intrinsic complexities of this type of investigation (11).

Vitamin supplementation studies in older adults have demonstrated a role for vitamin E in the production of IL-2 as well as the activation induced T cell proliferation in naïve but not in memory T cells (78, 113–115). However, this response is variable, depending on genetics and immune functionality (102). Moreover, age-related oxidative stress, hence inflamm-aging, can be counteracted by vitamin C supplementation. In addition, it seems that this vitamin is involved in enhanced antibody generation and in differentiation and maturation of immature T-cells as well as of NK cells. Because vitamin C is water-soluble and humans have low storage capacity, its regular intake is up to 100-fold higher than that for many other vitamins (116, 117).

Probiotics, Prebiotics, and Symbiotics

The use of probiotics, prebiotics and symbiotics, i.e., the combination of pro and prebiotics, as immunomodulators, which act on microbiota, is very common. However, no strong cause-effect relation often exists between their use and specific end-points. Gut microbiota that plays an active part in healthy status, is compromised in older adults due to malnutrition, use of medications and immunosenescence itself. Therefore, the administration of specific strains of *Lactobacilli* and *Bifidobacteria* as probiotics as well as fructooligosaccharides, galactooligosaccharides, and other prebiotics, or the combination of both might constitute a benefit for immunocompromised people (118–122).

Data from supplementation studies with pro- or prebiotics in older adults show a control of inflammatory status because their use is responsible for a lower production of TNF-α, IL-1β, and IL-6 as well as an increase of anti-inflammatory cytokine IL-10, by PBMCs. In addition, these biotics improve the innate immune responses by the modulation of phagocytosis and cytotoxicity against specific bacteria, such as *Staphylococcus aureus*, increase activity of peripheral blood NK cells, and lower CD25 expression by resting T lymphocytes (123). However, the complexity of randomized controlled trials and lack of specific biomarkers in humans make difficult the reproducibility of the data (124). Moreover, healthy status, including absence of disease and nutritional status, seems to be crucial for their action as demonstrated by null results on immunomodulation after administration of prebiotics in older adults vaccinated with influenza or pneumococcal vaccines (125). Further studies are summarized in a very recent review by Suez et al., although in this case too, it is highlighted the weakness of the existing data (124). Therefore, a strong limitation linked to the study of these potential modulators is the lack of mechanistic studies that could reveal the molecular mechanisms underlying their action. This would allow a targeted and effective use, and would reduce the bias linked to individual variability and the conflicting existing results present in literature. Although meta-analyses and systematic reviews report interesting data, they cannot replace multicenter, randomized controlled clinical trials to address the relevance of the use of probiotics or the composition of the microbiota, both accompanied by molecular explanation of the observed evidence.

Nutraceuticals

Recently, various bioactive food components associated with health-related effects have been called nutraceuticals. These food compounds, mainly found in plant-based foods and fatty fish, have been implicated in offering physiological health benefits over and above basic nutritional requirements (126–128). Now, there is much interest in optimizing the immune response, and in reducing inflammation in older adults by increasing the intake of certain bioactive food agents (129, 130). Many studies have investigated how immune function and inflammation are directly affected by nutraceuticals. They provide evidence that increasing intake of some of them above the habitual and recommended dose levels can enhance some aspects of immune function, and reduce the level of inflammatory status, increasing cellular resistance to aging (131–133). Below, we examine the immunomodulatory effects of three classes of nutraceuticals, namely carotenoids, polyphenols, and polyunsaturated fatty acids (PUFAs), summarizing the most relevant nutritional studies on the reciprocal interactions between these dietary agents and immunosenescence.

Carotenoids are naturally occurring pigments found in most fruits and vegetables. They primarily exert antioxidant, hence anti-inflammatory, effects, but individual carotenoids may also act through other mechanisms, including immune-enhancing activities (134, 135). Jyonouchi et al. observed that lutein and astaxanthin increased the *ex vivo* antibody response of mouse splenocytes to T-cell antigens (136). Older adults supplemented with carotenoids (30 mg β-carotene, 15 mg lycopene and 9 mg lutein) had a shift to T cells expressing a mature phenotype and, in addition, higher IgA serum levels, and an increase in NK cells (137). Watson et al. report that higher doses of β-carotene (30 and 60 mg/day; instead of 15, 30, and 45) increase T helper cells and NK cells number (138). Although higher doses of carotenoids are not easily achievable in the diet of population, these findings suggest that low doses are insufficient to affect immune responses. Enhanced NK cell cytotoxicity was observed in participants treated with oral β-carotene and, similarly, long-term β-carotene supplementation increased NK cell activity in older adults (139, 140).

Dietary polyphenols are the biggest group of phytochemicals and they are defined as bioactive non-nutrient plant compounds. They are in fruits, vegetables, grains, and other plant foods, the consumption of which has been linked to reduction in risk of major age-related diseases (141, 142). In fact, as discussed below, their main action is the control of inflammation. Consumption of cocoa polyphenols rich in flavonoids (40 g/day) with 500 ml of skimmed milk, by participants at high cardiovascular disease risk (≥55 years), significantly reduced the expression of cell adhesion molecule very late antigen-4, CD40, and CD36 on monocytes. This treatment also lowered circulating levels of the inflammatory markers P-selectin and ICAM-1, compared with monocytes from the control group (only skimmed milk) (143). *In vitro* studies have shown that administration of olive oil polyphenols (caffeic acid and oleuropein glycoside) to human whole blood cultures stimulated with lipopolysaccharides significantly reduced IL-1β levels compared with stimulated control cultures that were not incubated with olive oil polyphenols. Interestingly, responses were inversely correlated to the dose (144). A small scale ($n = 23$) pilot study has shown that daily consumption of 12 green olives, containing oleuropein and hydroxytyrosol, significantly reduced serum IL-6 and malondialdehyde (a lipid peroxidation marker) levels after 30 days of consumption by healthy adults (90). Although several reviews have postulated potential beneficial effects of polyphenols on the immune response of older adults, there have been limited studies on this topic (145). However, the major effects of polyphenols are associated with increased release of IL-2 and IFN-γ, hence enhancing immune response (146). For example, resveratrol, a polyphenol typically found in red wine, grape skins, and berries, induces a significant increase in T helper

cells and in the delayed-type hypersensitivity response of aged rats (147).

In addition to carotenoids and polyphenols, several studies have also shown that dietary lipids can modulate the immune response. Fatty acids that have this role include the long-chain PUFAs of the omega-3 (n-3) and omega-6 (n-6) classes. n-6 PUFAs, derived from plants and land animals, have minimal effects on immune response. n-3 PUFAs, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), found mainly in fish and fish products and in some plants (flax seeds), have the most significant impact on immune cells. These have anti-inflammatory properties inhibiting the formation of eicosanoids and synthesis of pro-inflammatory cytokines (IL-1β, TNF-α, IL-6), chemokines (IL-8, monocyte chemoattractant protein 1), and adhesion molecules (ICAM-1, VCAM-1, selectins) (148). However, because dose, timing of administration, and participant age are important modulating factors of the effect of these molecules, contrasting results exist and only few studies focus on their use in older adults (149–154).

CLINICAL APPROACHES CURRENTLY BEING INVESTIGATED

Growth Factors

The various aspects of IL-7 physiology raise the possibility that reduction of this pleiotropic cytokine level could contribute to the age-related decrease in the absolute number of thymocytes and naïve T cells. Therefore, IL-7 might be used as a therapeutic agent to enhance thymopoiesis in lymphopenic patients or in older individuals, so counteracting the first hallmark of immunosenescence, i.e., the reduction of naïve T cells. In fact, the profound structural remodeling that characterizes the thymic involution also affects thymic epithelial cells with a consequent reduction in the intrathymic production of IL-7 (155, 156).

IL-7 produced by thymic epithelial cells provides survival and proliferative signals to immature double negative $CD4^{-}CD8^{-}$ thymocytes and promotes V(D)J recombination of the T cell receptor (TCR) γ-c locus (157). Mutations in the IL-7Rα or γc in humans lead to severe combined immunodeficiency, confirming the importance of the IL-7 signaling pathway in the development of T cells (158, 159). At later stages, the IL-7/receptor signaling complex is required for the homeostatic proliferation of naïve T cells in the periphery, exerting a higher effect in the cytotoxic T cell subsets. The high expression of IL-7Rα on naïve T cells allows the maintenance of the pool of these cells, but there are limited amounts of IL-7 under physiological conditions. Following the encounter with its cognate antigen, naïve T cells lose IL-7Rα expression and differentiate into effector T cells. IL-7R down-regulation guarantees an efficient use of the limited amount of IL-7 to naïve T cells that need it, driving their proliferation and preserving their phenotype (160). IL-7Rα is re-expressed at the memory stage, ensuring cell survival and proliferation in memory T cell pool too (156).

Interestingly, IL-7Rα chain is an integral component of the receptor for thymic stromal lymphopoietin (TSLP). TSLP provides normally a co-mitogenic activity that is less potent than that of IL-7 (161). However, to best of our knowledge no study has been performed on the possible role of TSLP in the treatment of immunosenescence.

In the first clinical trial in humans, patients with metastatic cancer (age range 20–59 years) treated with different doses of IL-7 showed a dose-dependent increase in circulating $CD4^{+}$ and $CD8^{+}$ lymphocytes along with a decrease in Tregs (162). Since then, numerous other clinical trials have used the administration of IL 7 for treating patients with various malignancies and chronic viral infections. In HIV-infected patients, with persistently low $CD4^{+}$ T-cell counts despite viral suppression, repeated cycles of recombinant human IL-7 induced a dose-dependent increase in circulating levels of both naïve and memory $CD4^{+}$ and mostly naïve $CD8^{+}$ T cells (163).

Therefore, some data suggest that IL-7 could have a therapeutic potential in improving the clinical outcome in settings that require enhanced immunological responses. However, in the complex scenario of aging, the immunorestorative properties of IL-7 may not be as great as initially hoped, most probably due to the deterioration of the thymus structure. The integrity of cortical and medullary thymic architecture and the presence of functional thymic epithelial cells are required to support and maintain thymopoiesis (164). Therefore, IL-7 effect on T cell development probably should require the preceding restoration of the thymic architecture.

Checkpoint Inhibitors; The Example of PD-1 and CTLA-4

The role of the immune checkpoint inhibitors, MoAbs that inhibit the expression of certain proteins made by T cells and some cancer cells, or antibodies that block the activation of inhibitory receptors, are pivotal for the management of cancer that occurs both in young and old patients. In fact, immune checkpoint inhibitors promote the immunological control of cancer cells by blocking the immune inhibitory responses that are evolutionary designed to prevent continuing immunological responses once an antigenic stimulus has been eradicated (165). However, there is a gap in the knowledge of the role of immune checkpoint inhibitors in the control of immune response in older patients because the data from randomized clinical trials are conflicting and often lack adequate statistical power.

The PD-1 and the cytotoxic T-lymphocyte antigen (CTLA)-4 are examples of checkpoint inhibitory receptors. The first regulates the inhibition and the fine-tuning of T cell responses. The second is a protein that contributes to the suppressor function of Tregs, mediating the inhibitory effect through the coordinated actions with the co-stimulatory receptor CD28. Activation of CD28 induces on lymphocytes and monocytes the expression of PD-1, which in turn interacts with its ligand (PD-L1) to regulate the balance between stimulatory and inhibitory signals needed for effective immune responses against antigens. This engagement leads to the inhibition of $CD28^{-}$ mediated co-stimulation, hence of TCR-mediated lymphocyte proliferation and cytokine secretion. The modulation of these pathways boosts anti-cancer immunity. Interestingly, the expression of PD-1

increases on T cells of older adults and its blockade partially restores T cells to functional competence (166–168).

The studies we discuss below are clinical studies based on the response to cancer. However, positive clinical data mean an increase in effector cell immune response, i.e., that therapy is in some ways targeting immunosenescence, or at least, dealing with the consequences of immunosenescence. In fact, immunosenescence influences the efficacy of the immune checkpoint inhibitors in older people (169); accordingly, the therapy is less efficient in patients ≥75 years (see below), probably due to a greater degree of immunosenescence. Consequently, there is limited evidence of successful therapy with immune checkpoint inhibitors in older adults, although a few observations of effectiveness in some patients are very encouraging. In the metastatic melanoma, for example, the use of the MoAb Nivolumab, a PD-1 inhibitor, alone or in combination with other antagonists, has survival benefits independently on age (170, 171). In another study, the administration of PD-L1 antibody Atezolizumab also shows positive results for all participants enrolled (172). In these studies, T cells of older adults were still able to respond to the blockade of their inhibitory receptors with a recovery of cytotoxic activity. Moreover, there is evidence about the efficacy of anti PD-1/PD-L1 MoAbs in older patients with non-small cell lung cancer (NSCLC) compared with chemotherapy. The benefit of immunotherapy in terms of response is stackable between younger and older patients (173).

Regarding the CTLA-4 use, several preclinical and clinical trials have reported the role of CTLA-4 inhibition in some kinds of cancer. In particular, the blockade with Ipilimumab can establish an anti-leukemic effect after allogeneic hematopoietic stem cell transplantation and can restore anti-tumor reactivity for patients with relapse (174). Although durable responses were observed, the efficacy of CTLA-4 inhibition needs to be confirmed. However, a recent meta-analysis analyzed the contextual administration of anti-CTLA-4 (tremelimumab and ipilimumab) and anti-PD-1 (nivolumab and pembrolizumab) molecules in four different settings: melanoma, prostate cancer, renal cell carcinoma, and NSCLC. The authors demonstrated a 37% reduction of the risk of death in favor of immune checkpoint inhibitors compared with control arm (175).

Recently, it has been demonstrated that the efficacy of the treatment with immune checkpoint inhibitors can be influenced by the composition of the host gut microbiota (176). As discussed above, the gut microbiota influences the immune system of the host. In fact, the interaction between specific microorganisms molecular pathways and immune cells can regulate local or systemic inflammation, hence influencing immune response (177). In particular, in cancer patients, the gut microbiota dysbiosis, caused by broad-spectrum antibiotic use, can be a contributor to immune checkpoint inhibitors resistance. In one study of 249 patients with NSCLC, renal cell carcinoma, and urothelial carcinoma treated with MoAbs against PD-1/PD-L1, a shotgun sequencing identified an overrepresentation of bacterial genera including *Akkermansia muciniphila* in responders to PD-1 inhibition compared with non-responders. In these patients, lymphocyte reactivity against *A. muciniphila* and IFN-γ production was significantly associated with survival (178). The analysis of 112 buccal and fecal samples from patients with metastatic melanoma also showed that the response to anti-PD-1 therapy depends on differences in the diversity and composition of the patient gut microbiota of responders vs. non-responders (179). These data demonstrated that, in responding patients, there was a relative abundance of bacteria of the *Ruminococcaceae* family. Moreover, in mice and patients, T cell responses specific for *Bacteroides* species, such as *thetaiotaomicron* or *fragilis* were associated with the efficacy of CTLA-4 blockade. On the contrary, tumors in antibiotic-treated or germ-free mice did not respond to CTLA blockade (180). Moreover, fecal microbiota composition of 26 patients with metastatic melanoma, using 16S rRNA, at time 0 and before each Ipilimumab treatment, was clustered on microbiota patterns. Baseline gut microbiota enriched with *Faecalibacterium* and other *Firmicutes* was associated with beneficial clinical response to Ipilimumab (181).

With the advent of immune checkpoint inhibitors immunomodulation is going to revolutionize the clinical management of at least some forms of cancer in older patients. In spite of several controversial points, some clinical trials suggest a significant benefit of immunotherapy in older patients, with the exception of patients ≥75 years that obtain less benefit from these treatments. Concerning this point, Metcalf et al. (25) have demonstrated that $CD28^-$ costimulation is required for the expansion of $PD\text{-}1^+$ CD8 T cells and effectiveness of PD-1 therapy in murine models of chronic viral infection and cancer. In addition, in lung cancer patients, PD-1+ CD8 T cells that proliferate in the peripheral blood after PD-1 blockade express CD28. Therefore, these data, which imply selective proliferation of $CD28^+$ cells by PD-1 therapy, highlight one mechanistic explanation why cancer patients older than 75 years may not respond as well to immunotherapy as younger patients. Understanding immune-regulatory functions is critical to implement integrative immunomodulatory strategies targeting checkpoints inhibitors.

Further studies of these checkpoints inhibitor functions might provide to be of great therapeutic value also in improving T cell responses to boost anti-microbial immunity and vaccine efficacy during aging as well. The combination of immunological, biochemical and systems biology data provides significant support for using PD-1 as an important target for therapeutic interventions of this type. In fact, studies carried out on HIV, hepatitis B and hepatitis C infections have shown that blocking the interaction PD-1/PD-L1 has a positive effect on the effector functions of T cells. Furthermore, future studies focusing on the elucidation of additive effects of blocking PD-1, other negative regulatory molecules, and immunosuppressive cytokines will help to identify combinatorial approaches that can improve T effector responses to vaccination and therapeutic interventions in older patients (182).

MAPK Pathway; Focus on p38 Regulation

Recently, the role of MAPKs pathways in the functional competence of the immune system has been demonstrated (183). The MAPK signaling pathways have been extensively studied in the context of oncogenic function and proliferative stimulus. However, these complex systems also regulate several

functions of the innate and acquired immunity. They are also involved in the production of pro-inflammatory cytokines, as well as in the intracellular signaling cascades initiated when a cytokine binds to its corresponding receptor (183). Three main subgroups of MAPKs are known: Erk, Jnk, and p38. These kinases can be targeted by small molecular weight compounds, which act to inhibit the phosphorylation of proteins, hence preventing their activation. Each one is separately regulated within individual cells (184) [for an overview of kinase inhibitors see (183)]. Understanding the immune-regulatory functions exerted by MAPK pathways is critical to implement integrative immunomodulatory strategies targeting these kinases.

The p38-MAPK pathway plays a pleiotropic role in cell survival, both sustaining proliferation, and inducing apoptosis in a cell type-specific manner, depending on the type of stimulus (185). The p38-MAPK pathway stimulates the positive regulation of Th1 differentiation and polarization. This pathway is not active in Th2 cells (186). The p38-MAPK pathway is critical for the production of inflammatory cytokines, positively regulating the production of IFN-γ in CD4 and CD8 cells (187, 188).

The studies discussed below have been performed *ex vivo* in mononuclear cells from mice and humans. They point out the possibility to affect the second hallmark of immunosenescence (the accumulation of memory T cells) through the regulation of p38 activation. p38 is generally absent in senescent human T cells. However, IFN-α signal can activate p38, triggering cellular senescence, and leading to inhibition of proliferation and telomerase activity in non-senescent T cells (189). It is also associated with alterations of energetic metabolism as well as autophagy. Autophagy, by inhibiting cell senescence, is a critical regulator of memory $CD8^+$ formation, and age-related autophagy defect is one of the explanations why $CD8^+$ T memory formation becomes defective in old age (38, 185). In 2009, Eisenberg et al. identified the use of spermidine, a polyamine compound, to promote longevity, via autophagy, using PBMCs as model. The authors monitored the survival cells using annexin V/7-AAD as co-staining. After 12 days, 50% of the cells survived after addition of spermidine. The rescuing effect did not involve inhibition of apoptosis, as the percentage of apoptotic cells was not influenced by spermidine. In fact, cell death, associated with membrane rupture, was indicative of necrosis (190). In immunosenescence models, $CD8^+$ T cell can be also rejuvenated in an autophagy dependent manner, using spermidine (191, 192). Low doses of a synthetic compound of natural spermidine significantly suppressed autophagy in human Jurkat T cell line. Moreover, the use of spermidine dramatically improved the $CD8^+$ T cell response to vaccination and infection in aged mice in an autophagy-dependent manner, contributing to the increased numbers of antigen-specific $CD8^+$ T cells (191).

Moreover, the effector memory $CD8^+$ T cells that express CD45RA, are not functionally exhausted. Indeed, they preserve the ability to secrete high levels of specific cytokines, such as IFN-γ and TNF-α. Furthermore, they only express low levels of key markers of exhaustion, such as PD-1. In these cells that present characteristics of immune senescence (decreased proliferation, lower telomerase activity, and increased presence of DNA damage), the simultaneous blockade of both p38-MAPK and PD-1 signaling supports their proliferation, both in young and in older human beings. Secretion of TNF-α in some populations of cells is reduced because of the contemporary arrest of p38-MAPK and PD-1 pathways. However, the telomerase activity in $CD8^+/CD45RA^+$ T cells is improved by blocking only the p38 pathway but not the PD-1 signaling, indicating that non-overlapping signaling pathways are involved (193, 194).

In addition to the inflammatory pathways that activate p38 through MAPK cascade by auto-phosphorylation, p38 can be associated with AMPK complex in response to chronic antigenic stimulation (see below, next paragraph).

The success of the studies using MAPK inhibitors, and kinase inhibitors in general, allows the possibility to analyze, and discover, the potential of these molecules in the treatment of immunosenescence, targeting the second hallmark. For example, a block at the level of p38-MAPK by sestrins causes age-related signaling defects in effector and memory $CD45RA^+/CCR7^-$ T cells (195, 196). Sestrins, the mammalian products of the Sesn1, Sesn2, and Sesn3 genes, are a family of stress sensing proteins (196). Lanna et al. proposed a possible role for sestrins in the control of the immune response, although this role has not yet been fully determined. Sestrins exhibit pro-aging activities in T senescent lymphocytes. The authors identified a complex named sestrin-dependent MAPK activation complex (sMAC) that simultaneously coordinates the activation of each MAPK that controls a functional response. The knockout of sMAC restored T cell activity (antigen-specific proliferation and cytokine production) from older humans, and enhanced responsiveness to influenza vaccination in the aged mice (196).

Examples of Nutrient Signaling Pathways: AMPK and mTOR

The mechanisms exposed above are distinct from another sestrin-inhibitory complex, containing GATOR and RAG A/B GTPase that involves the mTOR pathway (197–199). In particular, sestrins stimulate the activation of AMPK (by an unknown mechanism), inhibiting mTORC1 signaling. This suggests that the anti/pro-aging dichotomy of sestrin action in T cells vs. other cell types may depend on different sestrin-protein interactions (200).

In turn, senescent human $CD27^-/CD28^-/CD4^+$ T cells trigger AMPK to stimulate p38 recruitment, causing p38 auto-phosphorylation mediated by the protein scaffold TAB1. This pathway can inhibit telomerase activity, T cell proliferation, and expression of key components of the TCR signalosome. In the presence of low-nutrient levels and DNA-damage signaling the proliferative defect of senescent T cells is reversed by blocking AMPK-TAB1-dependent p38 activation (38). Moreover, in senescent $CD8^+$ T cells, p38-MAPK induces an increase in autophagy through interactions between a p38 interacting protein and autophagy protein 9, in a mTOR-independent manner, suggesting that p38-MAPK blockade reverses senescence via mTOR-independent pathway (185).

mTOR plays an important role in T cell activation and differentiation, especially of naïve $CD4^+$ T cells in their differentiation toward Th1 or Th17 phenotypes (201, 202). The

activation of mTOR signaling pathway is under the control of TCR/CD28 stimulation (201, 203). A growing body of research has highlighted mTOR inhibitors, i.e., rapamycin and everolimus, as promising treatments for several age-related pathologies, including immunosenescence, prolonging lifespan, especially in all four major animal models of aging: yeast, worms, flies, and mice (204, 205). The partial inhibition of mTOR could be beneficial for immune function in older people, although mTOR activity inhibits autophagy. At high doses, rapamycin is immunosuppressive, blocking both protein synthesis and cell division. In a clinical trial of over 200 older participants, they were assigned to a protocol including the use of mTOR complex 1 inhibitor everolimus, in different daily doses, for a 6-weeks period. Participants, after a 2-weeks drug-free interval, were challenged with the seasonal influenza vaccine. The two low-dose everolimus regimens improved immune function without causing serious side effects. Patients ameliorated their immune response, with improved hematopoietic stem cell function and a decreased proportion of PD-1^+ lymphocytes (206). In a subsequent follow-up study, combined BEZ235 (a dual ATP-competitive PI3K and mTOR inhibitor) and everolimus treatment for 6 weeks resulted in better infection control in older adults for a year after treatment had ended (207). However, rapamycin and Torin, another mTOR inhibitor, are also reported to suppress the anti-inflammatory effects of circulating glucocorticoids (208). These findings conflict with earlier studies showing the central importance of mTOR in innate immunity, specifically in the production of anti-inflammatory IL-10 and the suppression of pro-inflammatory cytokines IL-21 and IL-1β (209). The improved response after rapamycin treatment, which might involve a decrease in the percentage of PD-1 positive T cells, requires more detailed studies (207).

Data suggesting that nutrient signaling pathways may negatively influence lymphocyte function in aging indicate the possibility that inhibition of these pathways may enhance the activity of lymphocytes from older adults (210). Broad ranges of pharmacological agents with anti-immunosenescence properties have been identified and other trials with agents, such as rapamycin analogs are underway. Therefore, this represents a promising therapeutic approach to improving the health of older adults.

See **Figure 2** for the main clinical approaches in immunomodulatory interventions.

OTHER APPROACHES IN DEVELOPMENT

Other approaches focus on development of novel vaccines especially suited to raise protective immunity in older adults by overcoming the decrease in naïve cells. This approach includes high-dose vaccines, booster vaccinations, different immunization routes, and use of new adjuvant. The most used adjuvants are based on aluminum salts. These adjuvants induce the activation of APCs and strengthen the antigen immunogenicity by their slower release and higher persistence at the vaccination site. Another interesting compound is MF59, a squalene-based adjuvant, which increases the chemokine-dependent recruitment of APCs (211). However, adjuvants have shown only modest success (212). The most effective is generally considered complete Freund adjuvant, which can only be used in animals because it can cause a damaging skin inflammation (213). Therefore, there is an unmet need for new vaccine strategies for older people.

The development an identification of appropriate adjuvants and cytokines might effectively remedy defects in T cell functions from older adults, both directly and by better activation of DCs (214–216). Stimulation of TLRs by agonists seems to be a promising strategy to enhance vaccine efficacy, because TLR triggering can induce the production of cytokines by APCs, and can promote germinal center antibody production (217, 218). Age-related variations in cytokine production are seen in the APC isolated from older donors and efficient TLR stimulation may overcome the age associated TLR signaling dysfunction (219).

Triggering of multiple TLRs, using a combined adjuvant for synergistic activation of cellular immunity (CASAC), is an intriguing strategy. CASAC incorporates CpG (a single-strand oligodeoxynucleotide, characterized by motifs containing cytosines and guanines), which is a potent inducer of IFN-α by pDCs, in combination with polyI:C (a synthetic analog of viral dsRNA that targets TLR3, inducing the production of type I IFNs). CASAC also contains IFN-γ and MHC-class I and II peptides. This formulation results in potent cytotoxic T cell-mediated immunity in young mice. In fact, immunization with two or more TLR agonists, an activator anti-CD40 antibody, IFN-γ, and surfactants were sufficient to drive unprecedented levels of CD8 responses to peptides or protein antigens and highly polarized Th1 CD4 responses. CASAC stimulation activates both mDCs and pDCs with IL-12 secretion. This strategy is more effective than existing adjuvants and provides a technological platform for rapid vaccine development (213).

Accordingly, in aged mice, antigen specific $CD8^+$ T cell responses were stimulated after serial vaccinations with CASAC and a class I epitope, deriving either from ovalbumin or the melanoma-associated self-antigen, tyrosinase-related protein-2. Pentamer analysis revealed that aged, CASAC-vaccinated, animals had substantially higher levels of antigen specific $CD8^+$ T cells compared with mice vaccinated with complete/incomplete Freund adjuvant. Therefore, CASAC promoted significantly better functional $CD8^+$ T cell activity (220).

An approach able to overcome age-related defects in CD4 T cell responses *in vivo* comes from the ability of combined TLR ligands to induce the activation of peripheral blood DCs isolated from older healthy donors (29). Preliminary *in vitro* screening experiments suggest that, from the various TLR agonists tested, the condition that most effectively activates DCs is the combination of TLR7/TLR8 with TLR4. This TLR agonist combination induces significantly greater cytokine production than that induced by each of the individual agonists. This greater stimulation is probably due to the combined activation of both MyD88 and TRIF-dependent signal transduction pathways. Stimulation with the specific combination of TLR agonists, the imidazoquinoline R848 that targets TLR-7 and the monophosphoryl lipid A that targets TLR-4, induces significantly higher cytokine secretion by mDCs and pDCs from older

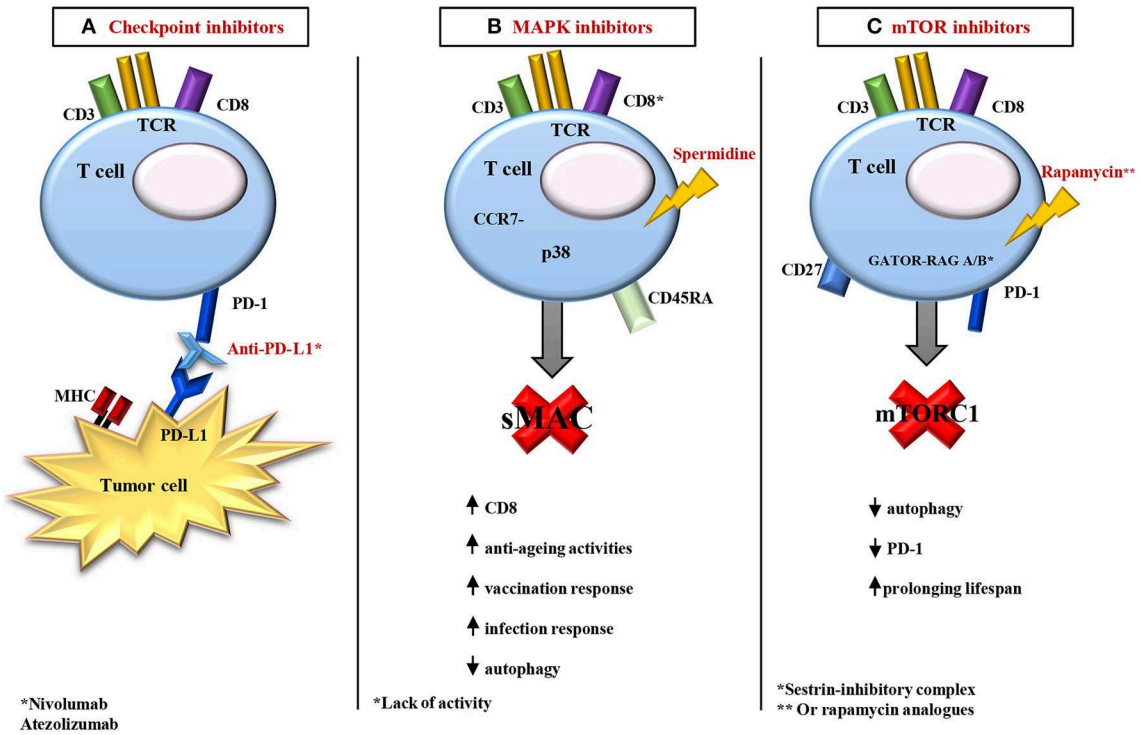

FIGURE 2 | Overview of the main strategies of immunomodulation. **(A)** In age-related diseases, in particular in some metastatic cancers, the use of the monoclonal antibody (such as Nivolumab, a PD-1 inhibitor) alone or in combination with other antagonists (PD-L1 antibody, like Atezolizumab) shows positive results on T cell activities in older adults. These cells became able to respond to the action of their inhibitory receptors with a recovery of cytotoxic activity. **(B)** In old models, with Lack of activity of $CD8^+$, it is possible to rejuvenate $CD8^+$ T cell responses in an autophagy dependent manner, using the polyamine compound spermidine. Spermidine induces autophagy and prolongs lifespan in model organisms. Moreover, in the $CD8^+/CD45RA^+/CCR7^-$ T cells, a block at the level of MAPK p38 by sestrins causes age-related signaling defects. The knockout of sMAC restores T cell activity (antigen-specific proliferation and cytokine production) in older humans, and enhances responsiveness to influenza vaccination in old mice. **(C)** GATOR and RAG A/B GTPase make a sestrin-inhibitory complex that involves the mTOR pathway. In particular, sestrins stimulate the activation of AMPK (by an unknown mechanism), inhibiting mTORC1 signaling. In addition, mTOR inhibitors, i.e., rapamycin and everolimus, are promising treatments for several age-related pathologies, including immunosenescence, prolonging lifespan. A soft inhibition of mTOR could be beneficial for immune function in older adults, although mTOR activity inhibits autophagy, and involves a decrease in the percentage of PD-1 positive T cells. See the text for the acronyms.

adults. This has potentially important implications, because the reduced production of cytokines by pDCs from older people, caused by defects in TLR signaling pathways, is associated with an ineffective antibody response to influenza vaccination (221). These findings highlight the efficient effect of adjuvants in the stimulation of cytokine production and point toward the potential use of appropriately selected combination of TLR agonists in future vaccination approaches for older adults to overcome the CD4 inability to respond.

CONCLUSION AND FUTURE APPROACHES

Until a few decades ago, a very small fraction of the population would reach 80 years of age. Now, in the Western world, this is a frequent event, with the average life expectancy for a newborn to have risen to 80 years in most Western European countries (1). However, the increase in lifespan does not coincide with increase in healthspan. The link between aging and disease is in part a reflection of the functional changes in the immune system of older people. Different factors contribute to the development of age-related immune dysfunction, but the epilog of an aged immune system is an increased propensity toward a reduced resistance to infection, poorer responses to vaccination, and the development of age-related diseases. The analysis of the contributing factors to this profound immune remodeling has revealed a complex network of alterations that influence both innate and acquired arms of the immune system. The diversity of cells, molecules and pathways involved in this remodeling, and their ability to influence each other, including the intra- and inter-individual variability of the immune response, make it hard to identify interventions that can be predicted to improve or, at least, to maintain the immune function in older adults. Within the past few years, numerous studies of the underlying mechanisms of age-related immune decline have laid the groundwork for the identification of targeted approaches; some of these have been discussed above, focusing on interventions able to target the hallmarks of immunosenescence. Possible other future approaches are reported below.

Taking into account the role of HCMV in the decrease of naïve T cells and increase of memory T cells, the reduction of the latent/lytic viral load, by vaccination and/or antiviral drugs, should be beneficial to diminish HCMV-associated

immunosenescence. Concerning the HCMV vaccine, Plotkin has published an extensive review. As pointed out by the author, as a result of 40 years of work, there are many candidate HCMV vaccines, including live recombinants, replication-defective virus, DNA plasmids, soluble pentameric proteins, peptides, virus-like particles and vectored envelope proteins. Therefore, we know the antigens needed in a HCMV vaccine, and that vaccination can be protective. To reach the goal of an effective HCMV vaccine, now we need a concentrated effort to combine the important antigens and to generate durable responses that will protect for a significant period. Interestingly, Plotkin emphasizes that aside from the two main targets for disease prevention, i.e., congenital infection and post-transplant disease, immunosenescence might be a target for vaccination mediated intervention, as well (222). Letermovir is an antiviral agent that inhibits HCMV replication by binding to components of the terminase complex. In patients undergoing hematopoietic stem cell transplantation, Letermovir daily prophylaxis is effective in preventing clinically significant HCMV infection when used through day 100 after transplantation, with only mild toxic effects and with lower all-cause mortality than placebo (223). However, there is no suggestion yet for the use of antiviral therapy as a strategy for prophylactic mitigation of immunosenescence.

Finally, possible future strategies to combat immunosenescence are represented by cellular and genetic therapies, including bone marrow transplantation and genetic reprogramming. In particular, genetically reprogramming cells into induced pluripotent stem cells can rejuvenate any cell type through telomere elongation, overcoming hurdles of replicative senescence (224).

SUMMARY

In the first part of the review we define immunosenescence and its relevance for the health of older persons, particularly in the context of acquired immunity. In the second part of the review we focus on the possible treatments to mitigate immunosenescence. First, we pay great attention to positive and negative effects of nutrition on immunosenescence. Then, we analyze the possible immunotherapeutic role of interleukin-7 as well as of checkpoint and mitogen-activated protein kinases inhibitors. Finally, we discuss a possible immunotherapeutic intervention to enhance the response of older adults to vaccines, i.e., the use of toll like receptor agonists. Therefore, we present a comprehensive review of several possible therapeutic interventions to alleviate immunosenescence.

AUTHOR CONTRIBUTIONS

AA, CC, SD, ML, and GA contributed to draft the manuscript. AA, FF, GC, CC, SD, CG, ML, NZ, and GA contributed to revising it. AA, CC, and GA wrote the final version.

FUNDING

The original research was funded by Italian Ministry of University (PRIN: progetti di ricerca di rilevante interesse nazionale—Bando 2015 Prot 20157ATSLF Discovery of molecular and genetic/epigenetic signatures underlying resistance to age-related diseases and comorbidities) to CC and GC. CASAC vaccination studies were also supported by UK Bloodwise (Programme Grant 13007—Pre-emptive immune therapy to prevent relapse of myeloid malignancies).

ACKNOWLEDGMENTS

Work in the Molecular Medicine Group at King's was supported by CRUK, the Experimental Cancer Medicine Centre, and the NIHR Biomedical Research Centres (BRC) based at King's Health Partners.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

ORIGINAL RESEARCH
published: 01 May 2020
doi: 10.3389/fimmu.2020.00827

Reduction and Functional Exhaustion of T Cells in Patients With Coronavirus Disease 2019 (COVID-19)

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Edited by:
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Specialty section:
This article was submitted to Immunological Memory, a section of the journal Frontiers in Immunology

Received: 21 March 2020
Accepted: 14 April 2020
Published: 01 May 2020

Citation:
Diao B, Wang C, Tan Y, Chen X, Liu Y, Ning L, Chen L, Li M, Liu Y, Wang G, Yuan Z, Feng Z, Zhang Y, Wu Y and Chen Y (2020) Reduction and Functional Exhaustion of T Cells in Patients With Coronavirus Disease 2019 (COVID-19). Front. Immunol. 11:827. doi: 10.3389/fimmu.2020.00827

Background: The outbreak of coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has posed great threat to human health. T cells play a critical role in antiviral immunity but their numbers and functional state in COVID-19 patients remain largely unclear.

Methods: We retrospectively reviewed the counts of T cells and serum cytokine concentration from data of 522 patients with laboratory-confirmed COVID-19 and 40 healthy controls. In addition, the expression of T cell exhaustion markers were measured in 14 COVID-19 cases.

Results: The number of total T cells, $CD4^+$ and $CD8^+$ T cells were dramatically reduced in COVID-19 patients, especially in patients requiring Intensive Care Unit (ICU) care. Counts of total T cells, $CD8^+$ T cells or $CD4^+$ T cells lower than 800, 300, or 400/μL, respectively, were negatively correlated with patient survival. T cell numbers were negatively correlated to serum IL-6, IL-10, and TNF-α concentration, with patients in the disease resolution period showing reduced IL-6, IL-10, and TNF-α concentrations and restored T cell counts. T cells from COVID-19 patients had significantly higher levels of the exhausted marker PD-1. Increasing PD-1 and Tim-3 expression on T cells was seen as patients progressed from prodromal to overtly symptomatic stages.

Conclusions: T cell counts are reduced significantly in COVID-19 patients, and the surviving T cells appear functionally exhausted. Non-ICU patients with total T cells counts lower than 800/μL may still require urgent intervention, even in the immediate absence of more severe symptoms due to a high risk for further deterioration in condition.

Keywords: SARS- CoV-2, COVID-19, T cell reduction, T cell exhaustion, cytokine strom

INTRODUCTION

In December 2019, a series of acute respiratory illnesses were reported in Wuhan, Hubei Province, China (1, 2). A novel coronavirus, initially named severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was identified as the cause of this disease by the Chinese Center for Disease Control and Prevention (CDC) (3). This disease, now designated as coronavirus disease 2019 (COVID-19) by the WHO, rapidly spread to other cities of China, and has become a public health emergency of international concern (PHEIC) following its global spread. COVID-19 clinically manifests as fever, cough, muscle pain, fatigue, diarrhea and pneumonia, and can cause death in severe cases (4–6). Up through March 20, 2020, China has reported 81008 cases of confirmed COVID-19 and 3,255 fatalities (7).

Since an effective immune response against viral infections depends on the activation of cytotoxic T cells that can clear infection by killing virus-infected cells (8), boosting the numbers and function of T cells in COVID-19 patients is critical for successful recovery. A recent study reported that the 82.1% of COVID-19 cases displayed low circulating lymphocyte counts (4–6). However, the factors which might cause the reduction in count, and the activation status of T cells in COVID-19 patients, remain uninvestigated. We retrospectively analyze here the clinical data from 522 cases of COVID-19 who were admitted into the General Hospital of Central Theater Command and Hanyang Hospital in Wuhan from December 2019 to January 2020. We also compare the expression of exhaustion markers PD-1 and Tim-3 on the surface of $CD4^+$ and $CD8^+$ T cells from COVID-19 patients and healthy controls. Our results thus provide a preliminary demonstration of T cell exhaustion during COVID-19 infection and suggest that more urgent, early intervention may be required in patients with low T lymphocyte counts.

METHODS

Patients

Medical records from 522 patients (aged from 5 days to 97 years) with confirmed COVID-19 and admitted into the General Hospital of Central Theater Command or Hanyang Hospital in Wuhan from December 2019 to January 2020, and 40 healthy people (aged from 2 to 62 years), who came to the hospitals for routine physical examination, were collected and retrospectively analyzed. Diagnosis of COVID-19 was based on the New Coronavirus Pneumonia Prevention and Control Program (5th edition) published by the National Health Commission of China (9). All the patients were laboratory-confirmed positive for SARS-CoV-2 by use of quantitative RT-PCR (qRT-PCR) of throat swab samples. This study was approved by the National Health Commission of China and Ethics Commission of General Hospital of Central Theater Command ([2020]-004-1) and Hanyang Hospital (20200217). Written informed consent was waived by the Ethics Commission of the designated hospital for emerging infectious diseases.

Definitions

The classification of clinical types, which consist of mild/moderate/severe/critical, was based on the New Coronavirus Pneumonia Prevention and Control Program (5th edition) published by the National Health Commission of China (9). Within the analyzed cohort, 43 patients were admitted to the intensive care unit (ICU), because they required high-flow nasal cannula or higher-level oxygen support measures to correct hypoxaemia. Hypoxaemia was defined as arterial oxygen tension (PaO_2) over inspiratory oxygen fraction (FIO_2) of $<$300 mm Hg or arterial oxygen saturation of 93% or lower. According to the staging of infectious disease (10), the prodromal period is a phase in which the host begins to experience general signs and symptoms. The illness period (overtly symptomatic period) is a phase in which the signs or symptoms of disease are most obvious and severe, with positive laboratory findings and chest/lung pathological manifestations. For ICU patients, ICU period is a phase in which the symptoms are most obvious and severe. The decline period is a phase in which the clinical symptoms begin to decline, laboratory findings and chest pathological signs improve, and arterial oxygen saturation normalizes.

Data Collection

We reviewed clinical records, nursing records, laboratory findings, and chest X-rays or CT scans for all the patients and physical examination records of the 40 healthy people. All information was obtained and curated with a customized data collection form. Three investigators (C Wang, Z Fen, and Y Chen) independently reviewed the data collection forms to verify data accuracy.

Sample Collection and Flow Cytometric Analysis

Peripheral blood samples from 14 patients and 3 healthy volunteers were simultaneously processed in the Central Lab of General Hospital of Central Theater Command to isolate peripheral blood mononuclear cells (PBMCs) for further testing. The peripheral blood was supplemented with anticoagulants (EDTA-K_2) and PBMCs were harvested by density gradient centrifugation. Isolated PBMCs were stained with a BD multitest IMK Kit (Cat340503, BD Biosciences) to analyze the frequency and cell number of total, $CD4^+$ and $CD8^+$ T cells, as well as B and NK cells in healthy controls and patients. The exhaustion of T cells was detected using human CD4-PerCP (RPA-T4, Biolegend), CD8-APC (SK1, BD Biosciences), CD8-PE (SK1, Biolegend), PD-1-PE (EH12.2H7, Biolegend), and TIM-3-FITC (F38-2E2, Biolegend) antibodies. After being stained, the cells were measured by flow cytometry on an LSR Fortessa Cell Analyzer (BD Biosciences) and data analyzed using the FlowJo software (TreeStar). All experimental procedures were completed under biosafety level II plus condition.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 8.0 (GraphPad Software, Inc., San Diego, CA, USA).

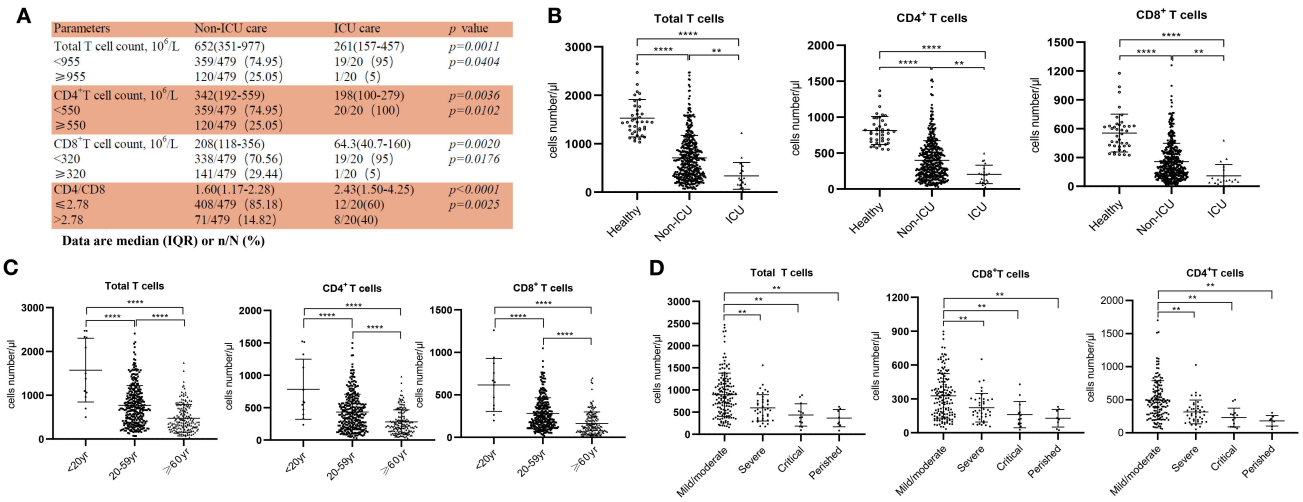

FIGURE 1 | Reduced T cell numbers in COVID-19 patients. **(A)** Demographics of T cells in patients; **(B)** T cell numbers in different groups; **(C)** T cell numbers in patients of different ages; **(D)** T cell count in Non-ICU care patients with different clinical outcomes. $^{**}p < 0.01$ and $^{****}p < 0.0001$.

Continuous variables were directly expressed as a range. Categorical variables were expressed as numbers/NUMBERS (%). Data in **Figure 2B** are analyzed using linear regression and R values are from Pearson's correlation coefficient test. *p*-values are from χ^2 (**Figure 1A**), non-paired *t*-test (**Figures 1A**, **2A**, **Supplementary Figure 1A**), paired *t*-test (**Figure 2C**, **Supplementary Figure 1B**), ordinary one-way ANOVA (**Figures 1B–D**, **3B,D**, **Supplementary Figure 1C**) and Pearson's correlation coefficient *t*-test (**Figure 2B**).

Role of the Funding Source

The funding agencies did not participate in study design, data collection, data analysis, or manuscript writing. The corresponding authors were responsible for all aspects of the study to ensure that issues related to the accuracy or integrity of any part of the work were properly investigated and resolved. The final version was approved by all authors.

RESULTS

Decreased Numbers of Total T Cells, $CD4^+$, and $CD8^+$ Subsets in COVID-19 Patients

From our retrospective analysis of 522 patients, 499 cases had lymphocyte count recorded. 75.75% (359/499), 75.95% (379/499), and 71.54% (357/499) patients had remarkably low total T cell counts, $CD4^+$ and $CD8^+$ T cell counts, respectively. Among milder disease patients in the Non-ICU group, the median value of total T cells, $CD4^+$ and $CD8^+$ T cell counts were 652, 342, and 208, respectively; the median value decreased to 261, 198, and 64.3, respectively, in the ICU group (**Figure 1A**). The counts of total T cells, $CD4^+$, and $CD8^+$ T cells were significantly lower in ICU patients than Non-ICU cases (**Figure 1B**). All these patients were further categorized into three groups based on age (<20 years old, 20–59 years and ≥60 years), and an age-dependent reduction of T cell numbers was observed in COVID-19 patients, with the lowest T cells numbers found in patients ≥60 years old (**Figure 1C**), suggesting a potential cause for increased susceptibility in elderly patients. It is worth noting that the age range of the ICU patients was 26–87 years [64.5 (53–70.75), Median (IQR)], suggesting that some young patients can become critically ill.

We next retrospectively reviewed T cell numbers in 212 cases from Non-ICU patients within one center (the General Hospital of Central Theater Command). The Non-ICU patients were further divided into four groups based on clinical outcomes. Among these patients, 151, 40 and 13 cases had mild/moderate, severe and critical disease, respectively, while 8 patient deaths occurred, in the perished group. Statistical analysis showed that T cell numbers including total T cells, $CD4^+$ and $CD8^+$ T cells in the severe and critical disease groups as well as the perished group were significantly lower than in the mild/moderate disease group. Most importantly, the numbers of total T cells, $CD8^+$ T cells and $CD4^+$ T cells in severe and perished groups were lower than 800, 300, or 400/μL, respectively (**Figure 1D**). This result suggests that there is a profound T cell loss in COVID-19 disease.

Negative Correlations Between T Cell Numbers and Cytokines

The expression of angiotensin converting enzyme 2 (ACE2), the predicted receptor of SARS-CoV-2 viruses, is absent on T cells (11), suggesting that the depressed T counts in COVID-19 patients mentioned above (**Figure 1**) were likely not caused by direct infection of T cells. We therefore examined the concentrations of serum cytokines, including TNF-α, IFN-γ, IL-2, IL-4, IL-6, and IL-10, from these COVID-19 patients to explore the influence of cytokine signaling. We only found the levels of TNF-α, IL-6, and IL-10 were significantly increased in infected patients, and statistical analysis illustrated that their levels in ICU patients were significantly higher than in Non-ICU patients (**Figure 2A**). It is also worth noting that cytokine levels

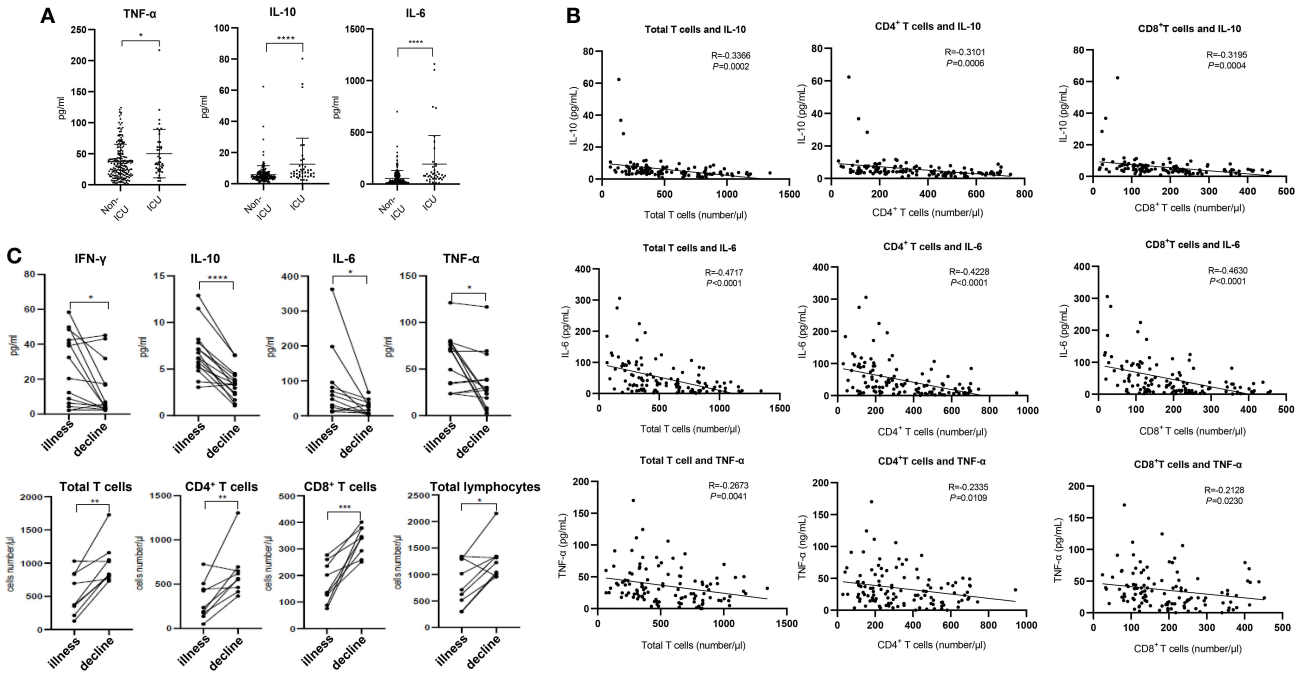

FIGURE 2 | Cytokines and relative T cell numbers in COVID-19 patients. **(A)** Cytokine levels in different groups; **(B)** Relationship between T cell numbers and cytokine levels; **(C)** Dynamic profiles of cytokine levels and T cell numbers in Non-ICU care patients. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

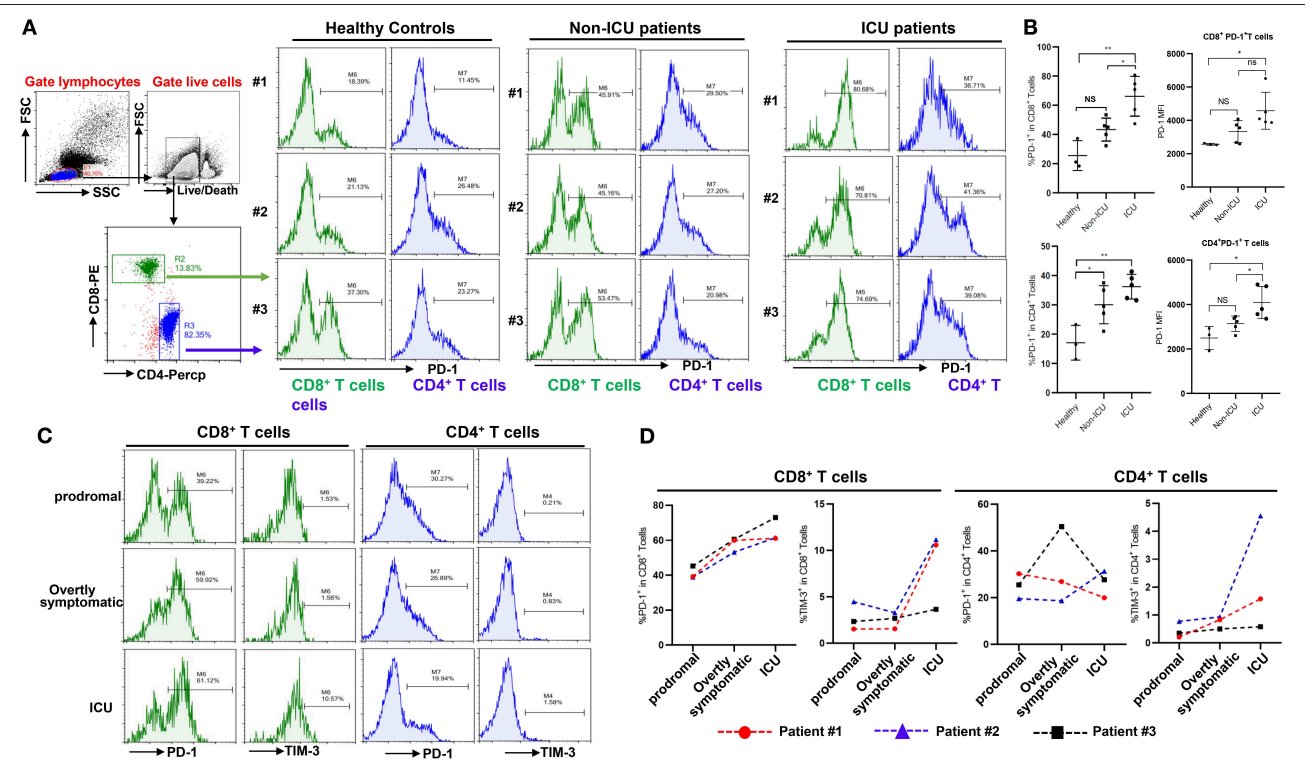

FIGURE 3 | Exhaustion of T cells in COVID-19 patients. **(A,B)** PD-1 expression on T cells in different groups; **(C,D)** Dynamic profile of PD-1 and TIM-3 expression on T cells in 3 patients. NS: not significant, *$p < 0.05$, **$p < 0.01$.

of some ICU patients were relatively normal, suggesting that a small minority of ICU patients were immunocompromised. The levels of IFN-γ, IL-2, and IL-4 showed no significant difference among different groups (**Supplementary Figure 1A**).

We next investigated the relationships between IL-10, IL-6, TNF-α, and T cell count within Non-ICU patients. Interestingly, the concentration of these three cytokines was negatively correlated with total T cell counts, $CD4^+$ counts, and $CD8^+$ counts, respectively (**Figure 2B**). We subsequently summarized the follow-up data of cytokine concentrations and T cell numbers in ten patients that were followed over the course of inpatient care. Interestingly, serum levels of IFN-γ, IL-10, IL-6, and TNF-α were significantly decreased in these patients in the decline (i.e., disease resolution) compared to the illness period, while counts of total T cells, $CD4^+$, and $CD8^+$ T cell subsets recovered during the decline period (**Figure 2C**). We also noted that serum levels of IL-2 and IL-4 had no difference between these two periods (**Supplementary Figure 1B**). The phenomena suggests that the decrease of T cells seen in COVID-19 patients may be the result of high serum concentration of TNF-α, IL-6, and IL-10 negatively regulating T cell survival or proliferation.

Progressive T Cell Exhaustion With Severity of COVID-19 Disease

Beyond changing in numbers during the course of infection, T cells may display limited function during prolonged infection as a result of exhaustion, which has been associated with the expression of some immune-inhibitory factors including PD-1, Tim-3 on cell surface (12). We therefore examined whether T cells in COVID-19 patients have exhaustion phenotypes. FACS analysis illustrated that, in comparison to healthy controls, especially ICU patients with COVID-19 disease showed markedly higher percentages of $PD\text{-}1^+CD8^+$ and $CD4^+$ T cells, with a trend to show higher PD-1 levels on $PD\text{-}1^+CD8^+$ or $CD4^+$ T cells (**Figures 3A,B**), indicating that SARS-CoV-2 can drive T cell exhaustion in COVID-19 patients, particularly in those requiring ICU care.

Three patients were followed during inpatient care, and the expression of the exhaustion markers including PD-1 and Tim-3 on surface of T cells during disease progress was detected. FACS analysis showed that these patients had very low percentages of $PD\text{-}1^+$ and $Tim\text{-}3^+$ on $CD8^+$ and $CD4^+$ T cells in the prodromal stage of disease, however PD-1 and Tim-3 expression tended to progressively increase in $CD8^+$ T cells during overtly symptomatic and ICU period disease stages (**Figures 3C,D**). Similarly, higher percentages of $Tim\text{-}3^+$ cells were observed in $CD4^+$ T cells from patients in the ICU stage, although PD-1 expression in $CD4^+$ T cells was not obviously affected during disease progression (**Figures 3C,D**). Furthermore, PD-1 and Tim-3 protein expression tended to increase throughout the stages discussed above in $PD\text{-}1^+$ or $Tim\text{-}3^+$ $CD8^+$ T cells (**Supplementary Figure 1C**). These results demonstrated that T cells are exhausted in COVID-19 patients during SARS-CoV-2 infection.

DISCUSSION

T cells play a vital role in viral clearance, with $CD8^+$ cytotoxic T cells (CTLs) capable of secreting an array of molecules such as perforin, granzymes, and IFN-γ to eradicate viruses from the host (13). At the same time, $CD4^+$ helper T cells (Th) can assist cytotoxic T cells and B cells and enhance their ability to clear pathogen (14). However, persistent stimulation by the virus may induce T cell exhaustion, leading to loss of cytokine production and reduced function (15, 16). Earlier studies have been unclear regarding the numbers and function of T cells in COVID-19 patients, albeit with suggestions of depressed lymphocyte counts (4, 6). In this report, we retrospectively reviewed the numbers of total T cells, $CD4^+$, $CD8^+$ T cell subsets in a total of 499 COVID-19 patients. In Non-ICU patients, we found that over 70.56% cases had a decrease in total, $CD4^+$ and $CD8^+$ T cells. However, in the ICU group, a total of 95% (19/20) patients showed a decrease in both total T cells and $CD4^+$ T cells, and most importantly, all of the patients displayed decreases in $CD8^+$ T cells. We also analyzed Non-ICU patients in greater detail, and found that urgent intervention may be necessary to preempt the development of severe symptoms in patients with low T cell counts.

Cytokine storm is a phenomenon of excessive inflammatory reaction in which cytokines are rapidly produced in large amount in response to microbial infection. This phenomenon has been considered an important contributor to acute respiratory distress syndrome (ARDS) and multiple organ dysfunction syndrome (MODS) (17, 18). It has been also implicated in the setting of respiratory viral infections, such as SARS in 2002, avian H5N1 influenza virus infection in 2005 and H7N9 infection in 2013 (19–22). Huang et al. showed that the levels of IL-2, IL-7, IL-10, TNF-α, G-CSF, IP-10, MCP-1, and MIP-1A were significantly higher in COVID-19 patients (4). Consistent with this report, here we found that the secretion of cytokines including TNF-α, IL-6, and IL-10 was increased in COVID-19 patients. Interestingly, the numbers of total T cells, $CD4^+$ T and $CD8^+$ T cells are negatively correlated to levels of TNF-α, IL-6, and IL-10, respectively (**Figure 2B**), suggesting these cytokines may be involved in the decrease of T cells detected in COVID-19.

TNF-α is a pro-inflammatory cytokine which can promote T cell apoptosis *via* interacting with its receptor, TNFR1, which expression is increased in aged T cells (23, 24). Our current analysis demonstrated that patients over 60 years old have lower T cell numbers, indicating that TNF-α might be directly involved in inducing T cell loss in these patients. IL-6, when promptly and transiently produced in response to infections and tissue injuries, contributes to host defense through the stimulation of acute phase responses or immune reactions. Dysregulated and continual synthesis of IL-6 has been shown to play a pathological role in chronic inflammation and infection (25, 26). Tocilizumab, a humanized anti-IL-6 receptor antibody, has been developed and approved for the treatment of rheumatoid arthritis (RA) and juvenile idiopathic arthritis (27, 28). Moreover, tocilizumab has been shown to be effective against cytokine release syndrome resulting from CAR-T cell infusion against B

cell acute lymphoblastic leukemia (29). Whether tocilizumab can restore T cell counts in COVID-19 patients by suppressing IL-6 signaling remains uninvestigated.

The source of these cytokines during COVID-19 disease remains an open interesting issue. While previous studies have validated that the secretion of cytokines, including IL-6, IL-10, and TNF-α, mostly derives from T cells, macrophages and monocytes etc. (30, 31), based on our (inverse correlation) results, we suggest that the secretion of these cytokines does not originate from T cells. However, the cytokine storm in turn may promote apoptosis or necrosis of T cells, and consequently leads to their reduction. Our previous work demonstrated that monocytes and macrophages can produce pro-inflammatory cytokine during murine hepatitis virus strain-3 infection (32, 33), yet whether SARS-CoV-2 also triggers cytokine release from monocytes and macrophages in COVID-19 patients needs further investigation and current work around this is in progress in our hospital.

T cell exhaustion is a state of T cell dysfunction that arises during many chronic infections and cancer. It is defined by poor effector function, sustained expression of inhibitory receptors, and a transcriptional state distinct from that of functional effector or memory T cells (34). By FACS analysis, we found that both $CD8^+$ T cells and $CD4^+$ T cells have higher levels of PD-1 in virus infected patients, particularly in ICU patients (**Figure 3**). IL-10, an inhibitory cytokine, not only prevents T cell proliferation, but also can induce T cell exhaustion. Importantly, blocking IL-10 function has been shown to successfully prevent T cell exhaustion in animal models of chronic infection (35, 36). We demonstrate here that COVID-19 patients have very high levels of serum IL-10 following SARS-CoV-2 infection, while also displaying high levels of the PD-1 and Tim-3 exhaustion markers on their T cells, suggesting that IL-10 might be mechanistically responsible. The application of potent antiviral treatments to prevent the progression to T cell exhaustion in susceptible patients may thus be critical to their recovery. We have read with great interest the successful application of Remdesivir to cure a COVID-19 patient in the US, and clinical trials indicate that this drug may have significant potential as an antiviral (37, 38).

Taken together, we conclude that T cells are decreased and exhausted in patients with COVID-19. Cytokines such as IL-10, IL-6, and TNF-α might be involved in T cell reduction. Thus, new therapeutic measures are needed for treatment of COVID-19 ICU patients, and perhaps these are necessary even early on to preempt disease progression in higher-risk patients with low T cell counts.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by National Health Commission of China and Ethics Commission of General Hospital of Central Theater Command ([2020]-004-1) and Hanyang Hospital (20200217). Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. Written informed consent was obtained from the individual(s), and minor(s)' legal guardian/next of kin, for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

YW and YC were involved in the final development of the project and manuscript preparation. XC and YZ wrote the manuscript draft. ZY, CW, and ZF analyzed the data. BD, YL, YT, LN, LC, ML, YL, and GW performed most of experiments.

FUNDING

This work was supported by the National Key Research and Development Program of China (No. 2016YFA0502204) and the National Natural Science Foundation of China (NSFC; Nos. 91442203, 81361120400, 91442203, 81471862, and 8177169).

ACKNOWLEDGMENTS

This manuscript has been released as a preprint at MedRxiv (39).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2020.00827/full#supplementary-material

Supplementary Figure 1 | Cytokines and Exhaustion of T cells in COVID-19 patients. **(A)** Cytokine levels in different groups; **(B)** Dynamic profiles of cytokine levels in Non-ICU care patients; **(C)** Dynamic profile of PD-1 and TIM-3 expressions on T cells in 3 patients. NS, not significant.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 30 June 2020
doi: 10.3389/fimmu.2020.01350

Cancer and HIV-1 Infection: Patterns of Chronic Antigen Exposure

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The main role of the human immune system is to eliminate cells presenting foreign antigens and abnormal patterns, while maintaining self-tolerance. However, when facing highly variable pathogens or antigens very similar to self-antigens, this system can fail in completely eliminating the anomalies, leading to the establishment of chronic pathologies. Prototypical examples of immune system defeat are cancer and Human Immunodeficiency Virus-1 (HIV-1) infection. In both conditions, the immune system is persistently exposed to antigens leading to systemic inflammation, lack of generation of long-term memory and exhaustion of effector cells. This triggers a negative feedback loop where effector cells are unable to resolve the pathology and cannot be replaced due to the lack of a pool of undifferentiated, self-renewing memory T cells. In addition, in an attempt to reduce tissue damage due to chronic inflammation, antigen presenting cells and myeloid components of the immune system activate systemic regulatory and tolerogenic programs. Beside these homologies shared between cancer and HIV-1 infection, the immune system can be shaped differently depending on the type and distribution of the eliciting antigens with ultimate consequences at the phenotypic and functional level of immune exhaustion. T cell differentiation, functionality, cytotoxic potential and proliferation reserve, immune-cell polarization, upregulation of negative regulators (immune checkpoint molecules) are indeed directly linked to the quantitative and qualitative differences in priming and recalling conditions. Better understanding of distinct mechanisms and functional consequences underlying disease-specific immune cell dysfunction will contribute to further improve and personalize immunotherapy. In the present review, we describe relevant players of immune cell exhaustion in cancer and HIV-1 infection, and enumerate the best-defined hallmarks of T cell dysfunction. Moreover, we highlight shared and divergent aspects of T cell exhaustion and T cell activation to the best of current knowledge.

Keywords: HIV infection, cancer, lymphocytes, cellular immunity, exhaustion, senescence, anergy, immune checkpoint

OPEN ACCESS

Edited by:
Alejandra Pera,
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Reviewed by:
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Specialty section:
This article was submitted to Immunological Memory, a section of the journal Frontiers in Immunology

Received: *09 February 2020*
Accepted: *27 May 2020*
Published: *30 June 2020*

Citation:
Vigano S, Bobisse S, Coukos G, Perreau M and Harari A (2020) Cancer and HIV-1 Infection: Patterns of Chronic Antigen Exposure. Front. Immunol. 11:1350. doi: 10.3389/fimmu.2020.01350

INTRODUCTION

The primary function of the human immune system is to protect the host by reacting upon the encounter of foreign antigens, as well as to prevent autoimmunity through self-recognition. Two arms orchestrate the activation of the immune system: the innate response triggered within the first hours and the adaptive response mounted over the following days, able to recognize and target

specific antigens and to generate memory. T cells are the major component of the adaptive immune system consisting of CD4 and CD8 T cells (1), being the latter key players in the physical elimination of tumor and virus-infected cells.

Most naïve T cells encounter their targets, presented by professional antigen presenting cells (i.e., dendritic cells, DCs), in secondary lymphoid organs (2). Such priming is crucial for determining the acquisition of functional attributes by T cells (3, 4). DCs govern the nature of primed T cells *via* the provision of processed antigens in the form of peptide/MHC complexes (signal I) and other important signals, including costimulatory interactions (signal II) and inflammatory cytokines (signal III) (5). Once activated, T cells undergo massive clonal expansion, differentiate into potent effectors, and express chemokines and homing receptors necessary for migration into peripheral tissues. Effector CD4 T cells produce several cytokines depending on the polarization determined by the cognate antigen and the extracellular milieu, effector CD8 T cells express cytotoxic molecules, such as perforin and granzymes, and produce effector cytokines. The production of cytotoxic molecules and cytokines is needed to help contain the spread of pathogens and tumors. The fate of naïve CD8 T cell differentiation is also determined by interdependent variables such as frequency of contact with the immunological synapses, epitope antigenicity, T cell receptor (TCR) affinity for cognate targets and the presence of CD4 T cell help (6). After CD8 T cell expansion and antigen elimination, any further immune activation is prevented by the upregulation and engagement of co-inhibitory molecules such as Cytotoxic T Lymphocyte Antigen-4 (CTLA-4) and Programmed Death-1 (PD-1). Most effector T cells die by apoptosis (contraction phase), but about 5–10% survive and differentiate into memory T cells. Different theories for memory T cell development have been suggested (7), but recent findings strongly suggest that long-lived memory CD8 T cells would arise from a subset of effector T cells through a process of dedifferentiation (8). Memory T cells are then maintained in the absence of antigens (homeostatic expansion) and can exert rapid effector functions in response to previously encountered antigens (1, 9).

Any disturbance of conventional activation signals may drive T lymphocytes to alternative cell fates, i.e., anergy, tolerance and exhaustion. This plasticity has evolved to constrain autoimmunity and excessive immune responses that would otherwise cause undesired tissue damage and immune-pathological conditions. Whereas, anergy is established during priming, due to the absence of costimulatory signals, and senescence is defined as growth arrest after extensive proliferation, exhausted T cells arise from cells which initially gained effector functions but became gradually dysfunctional due to continuous TCR stimulation by persistent antigens (10). Overlapping and discriminating functional and molecular features of these alternative cellular conditions have been comprehensively investigated (11, 12). In the present review, we describe the establishment and hallmarks of T cell exhaustion in HIV-1 infection and cancer. In addition, we highlight the parameters that allow the discrimination between functionally distinct T cell states, which are exhausted, activated, and memory T cells.

EMERGENCE OF T CELL EXHAUSTION

T cell exhaustion was initially described in the mouse model of LCMV infection (13–16), where, initially functional (17) and then transcriptional analyses led to the identification of PD-1 as first and main molecule associated with this status (15, 18, 19). Afterwards, high PD-1 levels have been observed in Simian Immunodeficiency Virus (SIV) infected Rhesus Macaques (15, 20–22) as well as in HIV-1 infected patients (23–25) and this was related to T cell impaired function and disease progression. In HIV-1 infection, T cell exhaustion is caused by antigen persistency and impaired CD4 T cell help (26, 27). During the acute phase of the infection, CD8 T cell responses are generated, but they are incapable of mediating complete virus clearance. HIV-1 is, indeed, endowed with a high mutation rate capacity that leads to a quick and efficient escape from immune cells (28, 29). Moreover, lymphoid follicles have been shown to be enriched in HIV-1/SIV-infected CD4 cells, and poorly infiltrated by CD8 T cells during early SIV infection. Consistently, the frequency of SIV-specific CD8 T cells entering the lymphoid follicles is inversely associated with the levels of infected cells, suggesting a new mechanism of viral persistency (30). While infected cells are not eradicated, T cells are continuously exposed to viral antigens, leading to a permanent expression of negative receptors and consequently to T cell dysfunction (15, 31–34). Of note, beside antigen escape, HIV-1 preferentially infects HIV-1-specific CD4 T cells (35), leading to profound consequences in the immune-pathogenesis of the disease (28). HIV-1-specific CD4 T cells expand at high frequency during the early phase of the infection. Later on, their number decreases in blood and secondary lymphoid organs (36), due to killing by HIV-1-specific CD8 T cells, virus cytopathic effects and pyroptosis triggered by abortive viral infection (37). In an early stage, CD8 T cell responses are also quickly impaired (27, 38–40), nevertheless this loss of function is partially restored in the presence of HIV-1 specific CD4 T cells (13, 27), highlighting the importance of CD4 T cell depletion in determining CD8 T cell exhaustion. CD4 T cells indeed provide help for CD8 T cells by producing supportive cytokines including interleukin (IL)-2 and IL-21, which can act directly on the responding CD8 T cells (41–48). IL-2 has a pivotal importance during priming of CD8 T cell response, in order to generate functional memory cells able to perform homeostatic turnover and to mount potent secondary responses (49). IL-21 instead has a major role in sustaining and expanding memory CD8 T cells (43, 44). In mice, CD4 T cell help has been recapitulated by CD27 agonism that enhanced specific CD8 T cell effector functions in response to vaccination or a viral infection (50).

Induction of T cell exhaustion is a common trait between HIV-1 infection and cancer (17), however key differences distinguish antiviral from anti-tumor immunity due to the pathogenesis of the two diseases (**Figure 1** and **Table 1**). The immunogenicity of the tumor is shaped by the immune system through a process called "immunoediting," as the pivotal work of Bob Schreiber first showed 15 years ago (116). In a first phase, the adaptive and the innate immune systems

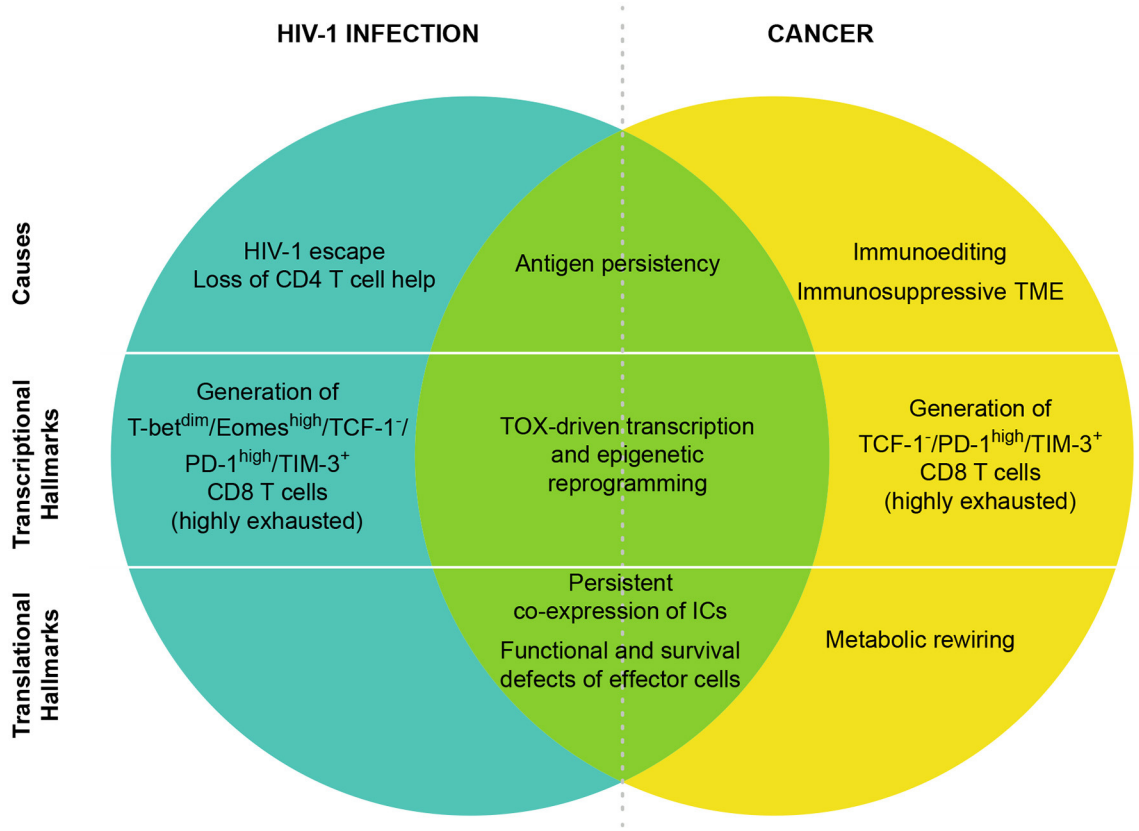

FIGURE 1 | CD8 T cell exhaustion in HIV-1 and cancer. T cell exhaustion in HIV-1 infection and cancer presents common origins and hallmarks, but also different features. The shared cause of T cell exhaustion is antigen persistency due to immune escape mechanisms. Moreover, in HIV-1 infection, the high viral mutational rate contributes to the immune escape while the preferential tropism of the virus for HIV-1 specific CD4 T cells leads to CD4 T cell loss that is also a main contributor of CD8 T cell exhaustion. In cancer, immunoediting and TME immunosuppression are peculiar determinants of tumor-specific CD8 T cell exhaustion. In both cancer and HIV-1 infection TOX has been identified as a master regulator of the transcriptional and epigenetic reprogramming of exhausted T cells. In HIV-1, T-bet^{dim}/Eomes^{high} subset defines highly exhausted CD8 T cells, however in CD8 T cells from cancer patients T-bet and Eomes are expressed in T cells with different levels of exhaustion. At the protein level, the co-expression of many ICs has been identified as hallmark of T cell exhaustion in both cancer and HIV-1 infection. Exhausted cells are also characterized by functional and survival defects including reduced effector functions, expansion capacity and increased susceptibility to apoptosis. Metabolic rewiring is also a key player of T cell exhaustion, however in HIV-1-infected patients little/no information is available so far.

synergize to recognize and eliminate malignant cells using conventional mechanisms (elimination phase). These include: the specific recognition of tumor-associated antigens and the expression of effector molecules by T lymphocytes (type I and II- interferon, perforin, Fas/FasL, tumor necrosis factor (TNF)-related apoptosis-inducing ligand—TRAIL), analogously to a viral infection, paralleled by the expression of recognition molecules such as NKG2D or ligands on tumor cells (induced by DNA damage and stress pathways) (117). Early infiltration of tumors by immune cells such as pro-inflammatory macrophages, both CD4 and CD8 T lymphocytes, NK, and DCs is crucial for tumor control (118–121). In the second phase, dormant tumor cells survive in equilibrium with the immune system where immunosuppressive and anti-tumor functions are balanced (equilibrium phase). In this phase, the tumor microenvironment (TME) is composed of several cell types that produce variable amounts of immune-suppressing and immune-stimulating molecules. In addition, tumors show a low proliferation rate and progressively undergo editing, resulting in tumor cell variants able to escape immune control (escape phase) (122). Clinically detectable tumors belong to this last and most studied phase with cancer cells proliferating with no or limited constraints. Tumor cells directly induce T cell exhaustion through the acquisition of somatic mutations, which confer increased immune resistance and survival, altogether contributing to prolonged antigen exposure. Ultimately, the exhaustion state is the outcome of a transcriptional and metabolic reprogramming induced by immunosuppressive cytokines (i.e., TGF-β, IL-10) and metabolites (i.e., lactate, kynurenine, adenosine, PGE2) produced by cancer cells (121, 123) and tumor infiltrating immunosuppressive cell subsets, including regulatory T cells, myeloid-derived suppressor cells, tumor-associated macrophages, cancer-associated fibroblasts, adipocytes, and endothelial cells (124). Moreover, anti-tumor T cells compete with cancer and immunosuppressive cells for nutrient availability and immunostimulatory factors. Current

TABLE 1 | Hallmarks of exhaustion.

IC expression	
HIV-1/chronic infection	**Cancer**
PD-1 (23–25)	PD-1 (51–56)
CTLA-4 (57)	CTLA-4 (53)
TIM-3 (34, 58–60)	TIM-3 (52, 53)
LAG-3 (61)	LAG-3 (53)
TIGIT (62, 63)	TIGIT (64–66)
CD160 (33, 67, 68)	CD160 (69)
2B4 (CD244) (67, 68)	2B4 (CD244) (70)
BTLA (60)	BTLA (71)
CD6 (72)	KLRG1 (73)
	VISTA (74–76)
	CD39 (77, 78)
	CXCL13 (53, 79)
	LAYN (80)
	Sia-SAMP:Siglec-9 (81)
Transcription factors expressed by exhausted CD8 T cells	
HIV-1/chronic infection	**Cancer**
Master regulators: TOX, TCF-1 (82–85)	
EOMES 187 (85)	STAT3 (86, 87)
BLIMP-1 (88–91)	BLIMP1 (55, 92)
TOX (93)	TOX (64, 93)
NOTCH (94)	NR4A2 (95)
NFATc1 (96)	NFAT (95)
BATF (97–100)	BATF (55)
IRF4 (100)	IRF4 (85)
VHL (101)	VHL (101)
FOXO1 (102)	FOXO1(102)
PBX3 (19)	FOXP1 (103)
c-Myb (85)	cMAF (104)
	GATA-3 (105)
	Zinc-dependent TFs (105)
Epigenetic of exhaustion	
HIV-1/chronic infection	**Cancer**
PD-1 locus demethylation was observed in models of chronic infections (106) and in HIV-1 infected patients (107)	Tumor-reactive makers CD39 and CD103 are demethylated in tumor-reactive CD8 T cells (whole-genome methylation profiling) (108)
Increased accessibility to *Pdcd, Havcr2,* and *Batf loci* and to *loci* encoding genes involved in negative regulation of T cell effector functions (109)	Recurrence after anti-PD-1 therapy was associated with the hypermethylation of the PD-L1 promoter (110)
Recent studies show the stability of the PD-1 locus demethylation even after PD-1 blockade (111)	Two chromatin states have been identified in exhausted T cells: (i) plastic and reversible, (ii) fixed dysfunctional state resistant to reprogramming (112)
Identification of exhaustion-specific enhancer that contains essential RAR, T-bet, and Sox3 motifs (109)	HDAC6-selective inhibitors directed peripheral and infiltrating T cells toward a Th1/effector phenotype (113)
Exhausted T cells acquire heritable *de novo* methylation programs able to restrict T cell expansion and clonal diversity during PD-1 blockade treatment. A DNA-demethylating agent (Decitabine) improved T cell responses and tumor control during PD-1/PD-L1 blockade (114)	
9–12 exhaustion clusters have been identified from epigenomic-guided mass cytometry profiling data (115)	

data suggest that nutrient deprivation is inducing, *per se*, T cell dysfunction (125–127).

CD8 T cell responses are quickly impaired during both early viral infection and tumor establishment.

Recently, terminally exhausted CD8 T cells have been characterized and distinguished from their progenitors depending upon the expression of PD-1, TIM-3, CD44, Eomes, T-bet, TCF-1, Slamf6, and CXCR5 (51, 67, 128–134). Exhausted T cell progenitors were characterized in LCMV models as pool of cells expressing TCF-1^{+}/PD-1^{int}/CXCR5^{+}/Slamf6^{+}, responding to PD-1 blockade and differentiating into terminally exhausted CD8 T cells (TCF-1^{-}/PD-1^{high}/TIM-3^{+}) (128–131). Their

presence was also described among circulating tumor-reactive CD8 T cells in melanoma patients and within TILs in primary melanomas (135) and non-small-cell lung cancer (NSCLC) (132). Interestingly, recent studies have better characterized a subset of $CD8^+/CXCR5^+$ T cells with proliferative capacity and able to infiltrate B cell follicles and inflamed tissues in the presence of chronic antigen exposure and inflammation (129, 131, 136–143). This subset shows heterogeneous phenotype and gene expression profile depending on the pathogenic context, still it is distinct from the $CXCR5^-$ counterpart pool and maintain cytotoxic properties (144). In addition of being part of the $TCF\text{-}1^+/PD\text{-}1^{int}$ progenitor pool (129, 145), these cells have been described as having variable levels of exhaustion and being similar to Tfh cells (20, 108, 129, 131, 141, 146–149). This is reflected in their capacity to help in the control of viral infection and of tumor growth, in the promotion of inflammation and in the induction of B cell responses (108, 136, 137, 144). The formation and maintenance of the $TCF\text{-}1^+/PD\text{-}1^{int}$ progenitor pool is orchestrated by the thymocyte selection-associated high mobility group box protein TOX. While TOX is a key player in the establishment of the exhausted state, its role is largely dispensable for the generation of effector and memory T cells. Antigen persistency is likely to be the cause of *Tox* induction since its expression is dependent on calcineurin and NFAT2. TOX is therefore the translator of persistent stimulation into a distinct T cell transcriptional and epigenetic developmental program leading to T cell exhaustion. TOX is also important for the subsequent differentiation into terminally exhausted cells that is counteracted and regulated by the phosphatase PTPN2 (82–84, 150–152). PTPN2 abrogation increases the number of terminally differentiated cytotoxic CD8 T cells promoting effective immune response, tumor/viral clearance and improved response to inhibitory molecules blockade (84). TOX induces genes that are important for the exhaustion precursor formation, including transcription factors (TFs) (e.g., *Tcf7, Nr4a2,* and *Tox* itself) and co-inhibitory receptors (e.g., *Pdcd1, Lag3, CD244,* and *Havcr2*). In conclusion, persistent activation and induction of TOX are common drivers of T cell exhaustion in both viral infection and tumor pathogenesis. However, specific players such as CD4 T cell loss and TME heterogeneity in infection and cancer, respectively, contribute to define distinct and overlapping traits of exhausted T cells in the two conditions.

HALLMARKS OF T CELL EXHAUSTION IN HIV-1 INFECTION

Many studies have indicated HIV-1-induced T cell exhaustion as main hallmark of the disease. Of note, HIV-1-specific CD8 T cells selectively show features of exhaustion as compared to bulk CD8 T cell populations and unrelated virus-specific T cells circulating in the same subject, as described in human and animal studies (153–155).

Expression of Multiple ICs

A complex network of stimulatory and inhibitory surface molecules orchestrates the functionality of CD8 T cells (156, 157). A cardinal feature of exhausted T cells in HIV-1 infection is the sustained expression of multiple inhibitory immune checkpoints (ICs) (**Table 1**).

The first and, to date, the most important IC involved in CD8 T cell exhaustion in chronic infections (15, 23–25, 52, 158) is PD-1. During chronic stimulation, PD-1 expression on virus-specific CD8 T cells is high and sustained (23–25, 68) because of mechanisms involving both TFs [i.e., T-bet (159), Blimp-1 (88, 160)] and soluble factors [i.e., IFN-α (161) and RANTES (156)]. In turn, PD-1 signaling affects the function, proliferation, survival and chemotaxis of CD8 T cells (23–25, 156, 162). *In vivo*, $PD\text{-}1^{high}$ SIV-specific CD8 T cells are characterized by a higher turnover (163).

The interaction of PD-1 with its two ligands PD-L1 and PD-L2 on hematopoietic and non-hematopoietic cells triggers the phosphorylation of two cytoplasmic domains and the subsequent recruitment of cytosolic tyrosine phosphatases Shp2 and Shp1, the TCR-phosphorylating kinase Lck, and the inhibitory tyrosine kinase Csk (164, 165). These effectors mainly act by antagonizing the CD28 costimulatory signaling (166–168) and the TCR signaling *via* dephosphorylation of SLP76 and ZAP70 (164, 166). Moreover, signaling molecules including ERK, Vav, PLCγ, PI3K, and Ras have been described as downstream targets of PD-1 signaling in T cells, leading to an impairment in metabolism, survival and cell growth (10, 165, 168, 169). PD-1 is also expressed by $CXCR5^+$ CD8 T cells (20, 170), a population particularly interesting for therapeutic purposes.

In addition, landmark studies in LCMV (67) and then SIV/HIV-1 infection (33, 34, 62, 171, 172) highlighted the relevance of multiple ICs co-expression (i.e., CD160, 2B4, TIM-3, T cell immunoreceptor with Ig and ITIM domains-TIGIT, CTLA-4 and LAG-3) to define deeply exhausted virus-specific CD8 T cells. The co-expression of multiple ICs may be due to their transcriptional co-regulation and non-redundant roles in the physiological control of CD8 T cell responses (130, 173–176). Increased disease progression, viral replication and lower CD4 T cell counts were directly associated with PD-1 (23), CTLA-4 (171), TIM-3 (58, 59), LAG-3 (61), and TIGIT (62, 63) expression. In addition, the superior proliferative capacity and the maintenance of cytotoxic functions by $CXCR5^+$ CD8 T cells concur with a lower surface expression of ICs and a higher expression of co-stimulatory receptors (CD28 and ICOS) as opposed to the $CXCR5^-$ counterpart (129, 131, 148). Of importance, SIV and HIV-1 specific CD8 T cell proliferation *in vitro* improves when distinct ICs (*i.e.* CD160, 2B4, TIGIT, BTLA, TIM-3) are blocked (24, 33, 60, 62) and administration of anti-PD-1 in SIV infected macaques (177–181) and HIV-1-infected patients (182) increases T cell immune responses, however clinical efficacy remains controversial (181, 183–190).

More recently, in SIV-infected macaques, the expression of CD6 by $PD\text{-}1^+$ CD8 T cells was associated with a reduced proliferation, cytokine secretion and cytotoxic capacity when compared to their $CD6^-$ counterpart. The frequency of $CD6^+PD\text{-}1^+$ CD8 T cells positively correlated with SIV viral load and combined targeting of CD6 and PD-1 effectively restored the

CD8 T cell proliferation capacity *in vitro,* suggesting that CD6 may be a new immunotherapeutic target (72).

Recently, the combination of transcriptomic and proteomic data allowed the identification of multiple cell clusters that were evolving with HIV-1 disease progression or initiation of ART (64). These data may lead to the understanding of new specific features of disease evolution and drive novel therapeutic approaches.

Alteration in TFs Expression and Epigenetic Regulation

Genomic approaches were recently applied to investigate the transcriptional profile of virus-specific exhausted CD8 T cells, revealing their unique molecular signature as compared to non-exhausted cells (**Table 1**) (19, 109, 111, 112, 115). Transcriptional analyses showed that exhaustion results from centrally connected pathways (19, 115, 191), having TOX as a master regulator. Indeed, TOX expression correlates with the presence of an exhausted phenotype during chronic infections in mice (LCMV) and humans (HCV) (82). In addition to TOX, several TFs coordinate gene expression networks, including PBX3, EOMES, BLIMP1 (*Prdm1*) (88–91), NOTCH (94), NFATc1 (96), basic leucine zipper transcription factor, ATF-like (BATF) (97–99), IRF-4, von Hippel–Lindau disease tumor suppressor (VHL), FOXO1, and FOXP1 (99–102, 130, 159, 192–198). At the molecular level, TCR stimulation leads to the induction of *Tox* expression (83) and induces the recruitment of TFs, like Notch (94), NFATc-1 (96), IRF-4 and BATF (100), at the promoter of different inhibitory receptors, ultimately driving their upregulation. Among the genes induced by TOX, *Tcf7* (encoding TCF-1) promotes the generation of exhaustion precursors through the induction of *Eomes* and *c-Myb* in early chronic infection, whereas PD-1 is needed to stabilize this pool (85, 199). IRF4 was also shown to favor CD8 T cell exhaustion while limiting memory T cell differentiation (100). Importantly, PD-1^{high}/Eomes^{high} and PD-1^{low}/T-bet^{high} T cells are both necessary to contain chronic LCMV infection (130). However, CD8 T cells presenting a T-bet^{dim}/Eomes^{high} profile represent a highly exhausted state with elevated levels of multiple inhibitory receptors (i.e., PD-1, CD160, and 2B4) (200, 201). In turn, PD-1 signaling reduces the expression of Bcl-xl (168), favoring the apoptosis of activated T cells (162, 202), and induces BATF leading to a decreased cytokine production, cytotoxic potential and proliferation rate of virus-specific CD8 T cells (97, 99). BATF induces the expression of T-bet and BLIMP-1 and correlates with PD-1 expression in murine models of chronic viral infection. BLIMP-1 is upregulated in patients with progressive, as opposed to non-progressive, HIV-1 infection (194, 203, 204) and is also associated with reduced T cell proliferation and effector-cytokine secretion capacity; however, these functions are restored by knocking down BATF or BLIMP-1 (88, 99). BLIMP-1 can also be induced in T cells upon priming with HIV-1 pulsed DCs together with other inhibitory molecules, including PD-1, TIM-3, LAG-3, and CTLA-4 (205).

The characterization of the epigenetic landscape of exhausted T cells gives novel and key insights to decipher the function of TFs. Comprehensive whole-genome analysis of chromatin accessibility (ATAC-seq) (206), has shown that exhausted CD8 T cells have a distinct epigenetic signature (95, 109, 111, 112, 207, 208). For instance, exhausted CD8 T cells have several chromatin regions with reduced accessibility (e.g., the *Ifng*, *Ccr7, Il7r, Nt5e, Tcf7,* and *Lef1 loci*), while presenting open chromatin regions in loci that govern the expression of IC molecules (e.g., *Pdcd1, Tigit, Ctla4)*, of ectoenzymes implicated in metabolic regulation (e.g., *Cd38, Entpd1*), of chemokines and cytokines (e.g., *Xcl1*) and of TFs (e.g., *Eomes, Ikzf2, Tox*) (64, 109, 111). The deletion of chromatin accessible regions including TF binding motif for RAR-retinoic acid receptor, T-bet, and Sox3 cause a dramatic reduction in PD-1 expression, demonstrating their important role in shaping exhausted T cell transcriptional profiling (64, 109, 208). Moreover, during chronic LCMV infection, the *Pdcd1* locus become completely demethylated (106), while the histone H3 is less diacetylated in CD8 T cells, indicating a loss in epigenetically active genes (209). In parallel, the transcriptional regulatory region of the PD-1 promoter is unmethylated in PD-1^{hi} HIV-1-specific CD8 T cells but not in donor-matched naive cells (PD-1^{-}) (107). Thus, in chronic LCMV (106) and HIV-1 infection (107), PD-1 expression in virus-specific CD8 T cells is controlled by the chromatin accessibility of the gene itself (epigenetic control) and by TF governing its expression (**Figure 2**).

Loss of Functions

Exhaustion in chronic viral infections has been described in both mice and humans as the progressive decrease in the capacity of virus-specific CD8 T cells to secrete cytokines, proliferate and exert cytotoxicity (23, 68, 210–213) as a consequence of persisting virus and antigen load (214). Loss of function characterizing exhaustion is hierarchical: IL-2 production is one of the first function to be extinguished, followed by TNF-α production, whereas the ability to produce interferon-γ (IFN-γ) is more resistant to inactivation (155, 213, 215–218).

Consistently with the hierarchical loss of effector functions by exhausted T cells, Riley et al. (219) demonstrated that such effector functions depend on the strength of PD-1 signaling, thus on PD-1 expression levels.

HALLMARKS OF T CELL EXHAUSTION IN CANCER

The identification of exhausted T cells in the cancer setting was inspired by previous knowledge gained in chronic viral infections. In human melanoma metastasis, T cells sharing many features of the exhaustion signature identified in LCMV infection were found (53). However, as discussed above, the establishment of exhaustion occurs differently in viral infection and cancer, the latter involving a complex network of players and mediators. The repertoire of tumor-specific T lymphocytes is generally devoid of highly avid autoreactive cells due to central and peripheral tolerance mechanisms, and priming may be inefficient due to the lack of co-stimulation, an inflammatory milieu and/or the presence of immunoregulatory cellular subsets (220). Therefore, a more heterogeneous pool of cells, fully

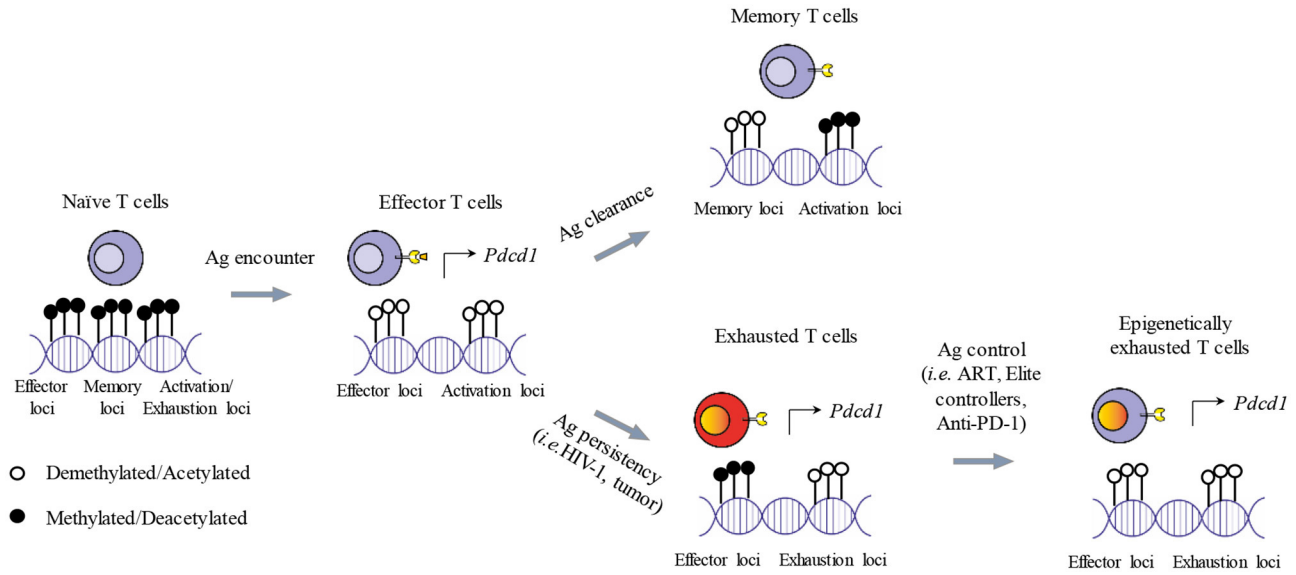

FIGURE 2 | Epigenetic imprinting of T cell exhaustion. Shortly after antigen exposure, naïve T cells generate effector T cells that are armed to eliminate foreigner antigens. Activated T cells demethlyate *loci* dedicated to the expression of effector functions and activation genes. Activated genes include also ICs (i.e., PD-1) needed for starting the contraction phase once the antigen is cleared. After the contraction phase, memory T cells survive and present a specific transcriptional profile, while PD-1 expression is reduced. If the antigen persists, memory T cells cannot be generated and effector functions are progressively lost. In addition, some genes of the demethylated *loci* remain transcriptionally active sustaining the expression of ICs and leading to T cell functional exhaustion. In the context of a therapeutic intervention or physiological immune control of chronic antigen exposure (i.e., ART, Anti-PD-1, Elite controllers), exhausted T cells can restore, at least partially, their effector functions and reduce the expression of ICs such as PD-1. However, implicated *loci* remain demethylated, potentially causing a rapid restoration of the exhausted state after treatment interruption and the failure of the immune system to completely eradicate the antigens.

activated or not, may undergo the dysfunctional program. Consistently with their virus-specific counterparts, these cells are characterized by increased expression of ICs (19, 64, 221), impaired homeostatic response to cytokines (222) and altered epigenetic and transcriptional programs (10, 191, 223). In contrast to HIV-1 infection where little/no information is available to date, the rewiring of the T cell metabolism in cancer immunopathogenesis is a well-characterized hallmark of exhaustion (224, 225). Of note, PD-L1 engaged by PD-1 acts as an anti-apoptotic molecule and increases chemoresistance on cancer cells through phosphorylation and activation of the PI3K/AKT pathway, as opposed to inactivation in T cells (226–228). Notwithstanding the recent burst of investigations on T cell exhaustion in cancer, studies in human remain challenging and animal models should be tuned to better reflect the slow course of natural cancer progression and its antigenic contexts (high/low mutational load).

Expression of Multiple ICs

In line with what is described for HIV-1 infection, a high and sustained expression of ICs is consensually considered as the main hallmark of T cell exhaustion in the cancer setting (**Table 1**). Tumor-specific CD8 TILs express high levels of PD-1 associated to impaired function (54). PD-1 is expressed upon TCR engagement and NFAT nuclear translocation (96) and may drive exhaustion of T cells undergoing persistent antigen exposure (18, 229). Exhausted T cells can co-express PD-1 together with different ICs, including, LAG-3, CTLA-4, BTLA, TIGIT, 2B4 (CD244), VISTA, KLRG1 (53, 73, 230) and TIM-3 (52, 131, 199). Inhibitory receptors signal through non-overlapping pathways and use different mechanisms to regulate T cell function ultimately inducing exhaustion: they sequester target receptors and ligands involved in activation pathways (ectodomain competition), they dampen the signals from activating receptors and they mediate transcription of inhibitory genes (10). Importantly, the hierarchical co-expression of multiple inhibitory receptors has been associated with a more severe grade of cellular dysfunction (231). Additional, recently identified markers of CD8 T cell exhaustion in cancer include: CD39 (77, 78), LAYN, whose expression is mutually exclusive with LAG-3 in hepatocellular carcinoma patients (80), and CXCL13 (53, 79). Moreover, Stanczak et al. (81) described the Sia-SAMP:Siglec-9 as an inhibitory pathway in NSCLC, where high frequencies of Siglec9$^+$CD8$^+$ TILs inversely correlate with survival (81). Finally, CD160$^+$ CD8 T cells have been shown to express higher PD-1 levels than the CD160$^-$ counterpart, to have less proliferative and cytotoxic potential and to be enriched among CD8 TILs in pancreatic cancer patients (69). Recently, a CXCR5$^+$ CD8 T cell population has been observed to expand in diffuse large B cell lymphoma (232), follicular lymphoma (144) and HBV-related hepatocellular carcinoma (137, 139, 141, 149). Circulating, tumor infiltrating, and lymphoid CXCR5$^+$ CD8 T

cells were shown to co-express PD-1 and, in contrast with chronic viral infection (129, 131, 134, 148), TIM-3 (134, 140), however they were functionally less exhausted than the CXCR5$^-$ CD8 T cell population and expressed genes related to stem-like plasticity and cytotoxicity (140, 141, 149). The frequency of this subset was correlated with a better prognosis in follicular lymphoma (144), pancreatic (139), colorectal (137, 141), and lung (140) cancer, suggesting its anti-tumor activity. However, combined blockade of TIM-3, PD-1 or IL-10R pathways could increase the cytotoxic activity of CXCR5$^+$ CD8 T cells indicating their limited lytic potential (139, 149).

Alteration in TFs Expression and Epigenetic Regulation

Tumor cells, together with immune and non-immune populations of the TME, contribute to a well-defined gene expression profile of dysfunctional anti-tumor T cells (**Table 1**), partially overlapping with that of exhausted T cells in chronic infections, by releasing molecules and establishing inhibitory contacts. In addition, recent studies in murine and human cancer suggest that TILs display a broad spectrum of dysfunctional states shaped by the multifaceted suppressive signals that occur within the TME (64, 130, 135). Several signaling pathways through the TCR, suppressive cytokines (TGF-ß, IL-6), inhibitory receptors, metabolites (adenosine, prostaglandins, lactate), enzymes (e.g., nitric oxide synthase, reactive oxygen species, indoleamine-2,3 dioxygenase), low pH, hypoxia and nutrient deprivation, lead to the final transactivation of TFs controlling the expression of different gene sets (101, 104, 173, 233, 234). As described for chronic infections, a complex pattern of TFs drives the initial triggering of differentiation toward the exhausted phenotype, including TOX, NFAT, Blimp-1, BATF, FoxO1, VHL, IRF4 (93, 234), Bcl-6, cMAF, and STAT3 (86, 87, 104, 235, 236). These factors exert distinct roles in T cells at different stages of differentiation and they do not exclusively govern gene expression in exhausted T cells. The epigenomes of different T cell subsets contribute to the context-specific functions of shared TFs. For instance, STAT3 dependent transcriptional regulation limits both TILs recruitment and cytotoxic function by downregulating IFN-γ, CXCR3, and CXCL10 expression and inducing ROR-γt (87, 236). Of note, EOMES and T-bet are expressed during the whole course of tumor progression and, in contrast to chronic viral infections, they do not help in distinguishing an exhausted-progenitor subset from terminally differentiated exhausted T cells (**Figure 1**) (10, 55). More recently, new technological advances (*i.e.*, mass cytometry and single cell sequencing) are allowing a deeper examination of the molecular properties of dysfunctional T cells at the single cell level. These studies represent milestones for the comprehension of T cell biology in the context of complex TME, dominated by a high heterogeneity of cellular subsets. Recently, Bengsch et al. (64, 115) identified 9 distinct T cell clusters among exhausted CD8 T cells in HIV-1 infection and human lung cancer by using transcriptomic- and epigenetic-guided mass cytometry. This study also assigned an exhaustion score to each of the subsets based on functional features (64, 115), those providing relevant insight for the design of IC blockade therapies.

Loss of Functions

As in chronic viral infections, exhausted T cells found in different tumor types have reduced effector functions as shown in terms of cytokine production and cytotoxicity (53, 237). Nevertheless, the hierarchy by which T cells progressively lose their functions is less clear (53, 54, 231, 237–239). TILs are not functionally inert and, to some extent, contribute to tumor control (231, 240). The efficacy of IC inhibitors and IL-2-driven *ex vivo* expansion of functional TILs is an indirect proof of this impaired yet present anti-tumor activity. Furthermore, TILs can be highly heterogeneous among distinct cancer types as evidenced by their different capacity to respond to IC blockade. For instance, in small-cell lung cancer patients, subsets of PD-1^{high} TILs are enriched in tumor-specific T cells and their presence is a predictor of clinical response to anti-PD-1 therapy (132, 241–243). On the contrary, T cells infiltrating breast tumor retain robust cytokine production and degranulation capacity (244) notwithstanding the expression of PD-1. In breast cancer patients, PD-1 expression is therefore less predictive of TILs dysfunction and this may explain the modest clinical responses to anti-PD-1 or anti-PDL therapies.

The proliferative potential of exhausted T cells is considered limited due to unresponsiveness to homeostatic cytokines such as IL-7, IL-15 and IL-21 (211, 245, 246). However, the previously mentioned TCF-1$^+$/ PD-1^{int} progenitor pool of exhausted T cells has a residual proliferative potential that allows the replenishment of the pool of exhausted antigen-specific CD8 T cells by expanding and differentiating into the numerically larger population of TCF-1$^-$/PD-1^{hi}/TIM-3$^+$ terminal progeny, characterized by a higher co-expression of other ICs and limited proliferative capacity (135, 199).

In a work by Li H. and co-workers, the intra-tumoral immune infiltrates of 25 melanoma patients differing for staging and treatments were analyzed by scRNA-seq for a deep characterization of dysfunctional T cells both in terms of transcriptional states and TCR clonality (238). Exhausted T cells expressing previously reported ICs (i.e., PD-1 and LAG-3) were observed in many patients. Importantly, intra-tumoral CD8 T cells could cluster in two distinct subpools. T cells belonging to the first pool spanned a wide range of transcriptional states, from transitioning to highly dysfunctional, expressed a gradient of inhibitory molecules and were specifically observed in tumor tissue. Some of the expressed regulatory molecules (CSF1, ZBED2) were also shared with regulatory T cells. A second subpool included T cells with cytotoxic potential, but limited proliferative capacity. This second pool of T cells could represent bystander T cells, likely from the circulation. Tumor-specific T cells were enriched in the exhausted pool, as previously observed for NSCLC (132). Strikingly, T cells with an initial buildup of the dysfunctional program maintained a clear proliferative signal with a doubling time of few days and rapid turnover. This dynamic and active T cell state fits previously suggested models of establishment of exhaustion at the tumor site (238). Common mechanisms of the emergence of exhaustion are present among

tumor types, but differences in the relative abundance of the subsets can be due to different TME, i.e., availability of antigens and exposure to inhibitory factors as well-shown for TILs in breast cancer (244). This is then reflected in the different capacity to respond to IC blokade that is not only heterogeneous among tumor types but also among individuals (244), as reviewed elsewhere (247).

In conclusion, in both HIV-1 infection and established tumors, T cell exhaustion is likely driven by TOX and the subsequent coordinated expression of several TFs. Exhausted T cells are characterized by loss of effector functions, high expression of multiple ICs, reduced homeostatic expansion, altered TFs expression, and remodeled chromatin. However, while in HIV-1 infection T-bet and EOMES allow the distinction between progenitors and fully exhausted T cells, in cancer patients TCF-1 and STAT3 may instead be the key TFs. The avidity and the hierarchy of the loss of function of exhausted T cells in cancer patients is less well-described than in chronic infections. Exhausted cells present in the TME may be highly heterogeneous and not include only the antigen-specific ones; new insights will explain how these aspects could affect the response to IC blockade.

EXHAUSTED VS. ACTIVATED/MEMORY CD8 T CELLS

Given the high heterogeneity and dynamicity of the memory CD8 T cell compartment (64, 238, 248), novel immunotherapies, aiming at rescuing the functionality of exhausted T cells, would require the ability to selectively distinguish exhausted from memory and activated effector T cells.

Expression of Surface Molecules

The solely qualitative evaluation of ICs expression by CD8 T cells, *per se*, does not discriminate between exhausted and activated T cells. As previously mentioned, inhibitory receptors that are transiently expressed on activated effector T cells show a higher and sustained upregulation on exhausted T cells, triggered by a persistent antigen stimulation. For instance, PD-1 is rapidly upregulated upon T cell activation (249) and persists at moderate levels in healthy subjects with a preferential expression on effector memory T cells (162, 250–253). During chronic infections, PD-1 expression on viral-specific T cells increases (23, 38, 128, 254, 255) and does not always reverse upon antigen removal (175, 256). HIV-1-infected patients responding to ART show reduced expression levels of PD-1 on virus-specific CD8 T cells after antigen clearance (257), still these levels are maintained above the physiological threshold observed in healthy individuals. This may be due to a broad systemic immune activation, to the effects of common gamma-delta chain cytokines sustaining PD-1 expression on bulk CD8 T cells (258, 259) or to the irreversible transcriptional and epigenetic alteration affecting highly exhausted T cells (106, 260) (**Figure 2**).

As previously mentioned, the degree of exhaustion is directly associated with the pattern of co-expression of different co-inhibitory receptors (67). First, this is mechanistically relevant, as simultaneous blocking of multiple ICs results in a synergistic reversal of T cell exhaustion in both cancer and chronic infections (171, 239, 261–263). Second, the identification of co-expression subsets may lead to a better discrimination between exhausted and activated T cells, reducing the risk for off-targets effects.

Many studies have shown that chronic antigen stimulation of T cells drives an IC expression pattern. For instance, TIM-3 and PD-1 cooperate for the induction of CD8 T cell exhaustion in cancer (52, 264–266) and chronic viral infections (34). In LCMV infection, PD-1 and TIM-3 identify a population of T cells strongly enriched in gene signatures of terminal exhaustion and harboring reduced proliferative capacity, longevity and cytokine production (64). In HIV-1 infected patients, ART significantly suppresses TIM-3 expression on HIV-1 specific CD8 T cells (267) indicating that, like PD-1, it is dependent on chronic TCR stimulation. Moreover, the expression profile of CD56 and TIM-3 can discriminate between individuals that naturally control HIV-1 replication (elite patients) and ART-treated patients (268). After virus-clearance and CD4 T cell recovery, patients receiving ART show a quantitative loss of $CD56^+$ CD8 T cells coupled to an exhausted phenotype, as shown by TIM-3 upregulation. Elite patients maintain a pool of cytolytic $CD56^+$ CD8 T cells comparable to healthy individuals. Similarly, CD160 expression also allows the distinction between exhausted and activated ($PD\text{-}1^+$) HIV-1 specific CD8 T cells (33). Indeed, only cells co-expressing CD160 and PD-1 ($PD\text{-}1^{high}CD160^{high}$) are functionally impaired in HIV-1 infected patients (33).

In cancer, the activation of ICs other than PD-1/PD-L1 and CTLA-4 can be induced by adaptive resistance to IC therapies. The treatment of such tumors could benefit from the combination of anti-PD-1 with different immune checkpoint molecules (e.g., LAG-3, TIM-3, TIGIT), activation markers and cytokines/chemokines (269). On the other hand, T cell dysfunction is characterized by decreased levels of co-stimulatory molecules, of their ligands and of adaptor molecules impairing the co-stimulatory signaling. Among these, CD44, LY6C, killer cell lectin-like receptor subfamily G member 1 (KLRG1), CD122 (IL-2Rβ), and CD127 (IL-7R), tumor necrosis factor receptor (TNFR)- associated factor 1 (TRAF1), CD28, and 41BBL have been described (67, 175, 246, 258, 259). In particular, exhausted T cells display the same profile of effector T cells with reduced telomere length and low levels of CD62L, CD127, and CD122 expression (1, 40, 153, 215, 258, 270–273). Their incapacity to respond to IL-7 and IL-15 (88, 128, 159, 245) lead to the lack of homeostatic expansion in the absence of antigens (1, 88, 128, 130, 159) and, ultimately, to death (14, 154, 215, 256, 274–276). T cell dysfunction is also characterized by the downregulation of the signaling adaptor TNFR-associated factor 1 (TRAF1) both in HIV-1 infected patients with progressive disease and in LCMV chronically-infected mice (259). In HIV-1 infected patients, TRAF1 expression negatively correlates with PD-1 expression and viral load and knockdown of TRAF1 in CD8 T cells from viral controllers results in decreased HIV-1 suppression *ex*

vivo. TGF-β is responsible for the post-translational loss of TRAF1, while IL-7 signaling is able to restore TRAF1 levels. Transfer of TRAF1$^+$ memory T cells or a combination treatment with IL-7 and agonist anti-4-1BB antibody in chronic LCMV infection improve T cell expansion and viral control in a TRAF1-dependent manner (259). Patient samples of renal cell carcinoma also show reduced expression of TRAF1 compared with normal kidney. This confers resistance to apoptosis and higher proliferative capacity to renal cancer cells (277). These findings identify TRAF1 as a potential biomarker of T cell dysfunction and therapeutic target. Moreover, combining PD-1 blockade with an agonistic antibody to 4-1BB dramatically improved T cell function and LCMV control *in vivo* (278). Still, the role of positive co-stimulatory molecules in rescuing exhausted T cells remains poorly described.

Transcriptional and Epigenetic Regulation

Another key difference between exhausted and activated T cells resides in the TFs (18, 19, 191, 279). Both the quality of the expressed TFs and the genes they can target, distinguish exhausted T cells from activated and memory CD8 T cells (191, 280, 281).

Transcriptional profiling analysis demonstrated that CD8 T cell memory and exhaustion reflect distinct states defined by coordinated sets of modules. Specific genes and pathways differentially implicated in exhaustion *vs.* memory include genes involved in epigenetics, DNA damage, and WNT signaling, such as *Rtp4, Foxp1, Ikzf2, Zeb2, Lass6, Tox*, and *Eomes* (191). The study by Bengsch et al. (64, 115) associates effector and exhausted T cells to a higher expression of CD39, LAG-3, TCF-1, Helios, CTLA-4 and PD-1, Eomes, TOX, 2B4, TIGIT, respectively.

During acute infection, T-bet and EOMES play pivotal roles in the generation of terminally-differentiated (2, 282) and central-memory (283–285) CD8 T cells respectively, while CD8 effector T cells co-express T-bet and EOMES (286). In contrast, during chronic infection, exhausted T cell subsets express either T-bet or EOMES in a somehow mutually exclusive pattern and they identify pools of non-terminal progenitor and terminally-differentiated exhausted CD8 T cells, respectively (**Figure 1**) (130). Of note, anti-PDL1 therapy only improves the function of the T-bet^{hi} subset, while having little impact on EOMES^{hi} cells (128, 130), indicating an important aspect of population dynamics in IC blockade-mediated reversal of T cell exhaustion. A similar population of CD8 T cells responding to IC blockade, (PD1^{int}/TCF-1$^+$), has been recently described as precursors of terminally exhausted cells (PD1^{high}/TCF-1$^-$/TIM-3$^+$) to be distinguished from memory precursors cells (PD1$^-$/TCF-1$^+$) on the basis of several epigenetic and transcriptional alterations such as higher expression of CXCR5 and Slamf6 (199).

By using a combined experimental and computational approach, Singer et al. (105) described mutually exclusive gene modules to distinguish dysfunctional from activated T cells in a murine colon carcinoma model. In particular, metallothionins, responsible for regulating the intracellular zinc metabolism, and zinc-dependent TFs were found to be highly enriched in dysfunctional CD8 TILs. GATA-3, a zinc-finger TF, consistently emerged as a driver of T cell dysfunction. Moreover, the expression of the co-inhibitory receptors PD-1 and TIM-3 was maintained upon metallothion-deletion, being uncoupled from the gene dysfunctional module (105).

Epigenetic studies also helped in identifying patterns distinguishing T cell exhaustion from T cell activation/memory profile. Recent epigenetic studies in mice and humans indicate that exhausted T cells represent a unique T cell lineage, compared to effector and memory T cells and are a stable, distinct and disease-relevant cell type (109, 111, 112).

HIV-1- and HCV-specific CD8 T cell genomes present a high accessibility to exhaustion-associated nucleotide regions. On the opposite, the genome of CMV-specific CD8 T cells is characterized by a higher accessibility to memory-specific nucleotide regions (109). Interestingly, the accessibility to exhaustion-specific regions is reduced in CD8 T cells specific for HCV epitopes that undergo viral escape (109), indicating that chronic exposure is needed to shape exhaustion-associated epigenetic imprinting.

Studies focusing on *Pdcd1* locus revealed that during the effector phase of an acute LCMV infection, the promoter regions were largely demethylated to become remethylated as the infection solved and CD8 T cell memory formed (106, 287). In the context of a chronic LCMV infection, the demethylation observed in the *Pdcd1* locus during chronic LCMV infection was instead stable and no remethylation was observed, even when viral titers and PD-1 protein expression by exhausted CD8 T cells decreased (106) or after transfer in recipient mice (260). Along the same lines, the unmethylated state of the *Pdcd1* locus did not change in T cells from subjects with a viral load controlled by ART for several years or from elite controllers (107). This suggests that the epigenetic program of the PD-1 locus is stabilized after prolonged exposure to HIV-1 virus despite different levels of PD-1 surface expression. Consistently, the transcriptome and the epigenome of terminally exhausted CD8 T cells (PD-1^{high}/TCF-1$^-$/TIM-3$^+$) are stably rewired and resistant to remodeling after PD-1 blockade (111, 114) (**Figure 2**).

These data strongly suggest that epigenetic remodeling may be required to further improve strength and breadth of the efficacy of immune checkpoint blockade.

In conclusion, exhausted T cells can be distinguished from activated T cells by the higher and sustained co-expression of IC molecules, as well as by a phenotype skewed toward effector memory cells with reduced co-stimulatory molecules expression. Moreover, the systemically induced immune activation and the stable transcriptional and epigenetic imprinting established during T cell exhaustion do not allow the restoration of IC molecules expression to the levels measured in healthy donors even after antigen removal/reduction. Among the TFs analyzed, T-bet, and EOMES allow the distinction between activated and exhausted CD8 T cells in HIV-1 infection, while metallothionins and GATA-3 have been suggested as discriminators in cancer patients.

CONCLUSIONS

Recent major advances in immunotherapy ultimately demonstrated the potentiality of the immune system in disease control. However, they also proved that existing strategies are hampered by the immune tolerance established by IC expression

on T cells. In addition, despite the significant difference in the availability of clinical information concerning immunotherapy efficacy in cancer and HIV-1 infection, there is still a long way to go for the scientific community to decipher the mechanisms of immunosuppression in different indications. Recently, new technological advances (such as mass cytometry, single cell sequencing, ATAseq, metabolomics) are allowing a deeper examination of the molecular properties of dysfunctional T cells at the single cell level. These studies represent milestones for the comprehension of T cell biology in the context of complex TME, dominated by a high heterogeneity of cellular subsets and in HIV-1 infection where current immunotherapy may not improve T cell responses (64, 115). These data may lead to the understanding of new specific features of disease evolution and drive novel immunotherapeutic approaches.

AUTHOR CONTRIBUTIONS

SV and SB wrote the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by the Swiss National Science Foundation (Grant 310030_182384).

ACKNOWLEDGMENTS

The authors acknowledge Mr. Samuel Cooper for his valuable proofreading of the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Immunology

REVIEW
published: 27 August 2020
doi: 10.3389/fimmu.2020.01915

To Remember or to Forget: The Role of Good and Bad Memories in Adoptive T Cell Therapy for Tumors

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The generation of immunological memory is a hallmark of adaptive immunity by which the immune system "remembers" a previous encounter with an antigen expressed by pathogens, tumors, or normal tissues; and, upon secondary encounters, mounts faster and more effective recall responses. The establishment of T cell memory is influenced by both cell-intrinsic and cell-extrinsic factors, including genetic, epigenetic and environmental triggers. Our current knowledge of the mechanisms involved in memory T cell differentiation has instructed new opportunities to engineer T cells with enhanced anti-tumor activity. The development of adoptive T cell therapy has emerged as a powerful approach to cure a subset of patients with advanced cancers. Efficacy of this approach often requires long-term persistence of transferred T cell products, which can vary according to their origin and manufacturing conditions. Host preconditioning and post-transfer supporting strategies have shown to promote their engraftment and survival by limiting the competition with a hostile tumor microenvironment and between pre-existing immune cell subsets. Although in the general view pre-existing memory can confer a selective advantage to adoptive T cell therapy, here we propose that also "bad memories"—in the form of antigen-experienced T cell subsets—co-evolve with consequences on newly transferred lymphocytes. In this review, we will first provide an overview of selected features of memory T cell subsets and, then, discuss their putative implications for adoptive T cell therapy.

Keywords: T cell, memory, adoptive T cell immunotherapy of cancer, competition, tumor immunity

OPEN ACCESS

Edited by:
Stefano Caserta,
University of Hull, United Kingdom

Reviewed by:
Karl Kai McKinstry,
University of Central Florida, United States
Roslyn Kemp,
University of Otago, New Zealand

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Specialty section:
This article was submitted to Immunological Memory, a section of the journal Frontiers in Immunology

Received: *20 December 2019*
Accepted: *16 July 2020*
Published: *27 August 2020*

Citation:
Mondino A and Manzo T (2020) To Remember or to Forget: The Role of Good and Bad Memories in Adoptive T Cell Therapy for Tumors. Front. Immunol. 11:1915. doi: 10.3389/fimmu.2020.01915

T CELL MEMORY AND ADOPTIVE T CELL THERAPY

T cells play a crucial role in immunity against pathogens and cancer. Antigen (Ag) encounter, costimulatory signals, and pro-inflammatory cytokines dictate naïve T cell (T_N) activation in secondary lymphoid organs, which is followed by clonal expansion and differentiation of effector T cells (T_{EFF}) (1–3). Although the vast majority of T_{EFF} cells die via apoptosis after antigen clearance, stable populations of memory T cells (T_M) can persist over time and ensure a rapid recall response upon further encounter with cognate Ag. The T_M pool is composed of several subsets harboring a considerable heterogeneity in trafficking, localization, effector functions, and durability; all features with direct consequences on recall responses.

The growing understanding of the cellular and molecular events underlying their behaviors, functionality, and persistence has instructed the development of defined T cell manufacturing protocols suitable for adoptive T cell therapy (ACT) against cancer and helped predict their behavior and efficacy *in vivo*. In the context of ACT, tumor-specific T_M lymphocytes are generally

produced *in vitro* by the expansion of tumor-infiltrating T cells (TILs) derived from tumor specimens or peripheral blood, or by the genetic engineering of peripheral blood mature T cells with tumor-specific T cell receptor (TCR) or chimeric antigen receptor (CAR). The adoption of ACT envisages several steps: (1) generation of T cell products, (2) conditioning of the host, (3) T-cell transfer, and (4) post-transfer cell support. Each of these steps can have a critical impact on ACT therapeutic efficacy, and vary according to infused T cells' T_M features, and simultaneously shape the immune landscape of the host. Indeed, mounting evidences indicate that the differentiation status of the transferred T cells along with tumor-intrinsic and tumor-extrinsic factors are important determinants of ACT clinical outcome (4). Once (re)infused in patients, tumor-specific T lymphocytes face the challenge to react to tumor lesions, which might vary in anatomical distribution and complexity, in the presence of a plethora of pre-existing T_M subsets, which might promote or oppose infused T cell activity. Although the density of $CD3^+$ TILs is generally a favorable prognostic factor for responses to therapy and overall survival of cancer patients, TILs can prove hyporesponsive or exhausted, and as such represent a barrier for ACT. Here, we review some of the seminal characteristics of memory/exhausted T cell subsets [reviewed in details elsewhere (3, 5, 6)] to highlight how pre-existing T_M might assist or outcompete newly transferred T cells, and by that represent an advantage or disadvantage for current ACT.

MEMORY T CELLS COME IN DIFFERENT FLAVORS

Although T_{EFF} cells mostly disappear upon pathogen/antigen clearance, T_M cells survive and patrol against secondary infection or metastatic recurrence in the case of tumors (7, 8). T_M cells consist of a collection of distinct subsets of cells with considerable heterogeneity in phenotype, function, location, and trafficking (9, 10). Based on distinctive migratory and effector properties, circulating memory CD4 T cells were initially classified in central memory T cells (T_{CM} cells) and effector memory T cells (T_{EM} cells) (11). CD4 T_{CM} cells, similar to T_N cells, express the lymph node and T cell zone homing receptors CD62L and CCR7 and produce substantial amount of IL-2, but lower levels of effector cytokines and cytotoxic molecules (11). A similar phenotype also characterized memory CD8 T cells. CD4 and CD8 T_{CM} cells have good proliferative capacity in response to Ag and ability to self-renew in response to IL-7 and IL-15. Within the long-lived memory subsets, also stem cell memory T cells (T_{SCM}) can be identified for their more naïve-phenotypic qualities and stem cell–like properties including the capacity to reconstitute the entire spectrum of memory and effector T cell subsets (12–15). The long-lived properties of both T_{CM} and T_{SCM} have been considered for effective vaccine design, and exploited in the setting of ACT, where they are associated with improved anti-tumor responses and therapeutic benefit. T_{EM} cells, instead, generally lack CD62L and CCR7, produce effector cytokines, and have higher cytotoxicity when compared with T_{CM}. Although T_{CM} circulate between secondary lymphoid organs and blood, T_{EM} circulate between blood and non-lymphoid tissues, where they persist long after Ag clearance (16, 17). The surface expression of the chemokine receptor CX3CR1 further refines T_{CM} ($CX3CR1^-$) and T_{EFF} ($CX3CR1^+$), (18) and identifies an additional peripheral memory T cell subset (T_{PM} $CX3CR1^{int}$), which appear to possess the highest steady-state self-renewal capacity of all T_M subsets, being able to survey peripheral tissues and return to secondary lymphoid organs, via the lymphatic system. A further distinct subset of T_M is constituted by tissue resident memory T cells (T_{RM} cells), which represent the front-line defense in case of reinfection, especially at barrier sites, such as the skin, lung, and gut, having rapid proliferation potential and immediate effector function capacity, (19–21) critically important in cancer immunology (22, 23). These have been described among CD4 and CD8 T cell subsets as being able to remain positioned within non-lymphoid tissues after Ag clearance and lack recirculation capacities (16, 17, 24). CD8 T_{RM} were initially characterized the by expression of CD103 [αE(CD103)β7], CD49a (VLA-1 or α1β1), and the C-type lectin CD69, critical for their retention into tissues, (25, 26) and for recruitment within epithelial tumor regions (27). More recently, data have shown that some CD8 T_{RM} cells lack CD103, and that this integrin is not an absolute marker for residency of CD4 T_{RM}, which also appear more heterogeneous compared with CD8 T_{RM} (28–30). Although the origin of CD4 T_{RM} cells remains debated, recent evidences suggest they might originate from CD4 T follicular helper subsets, which share with CD8 T_{RM} some key features related to their migration, differentiation, and maintenance [reviewed in (31)]. Lastly, it is worth mentioning that also Ag-inexperienced T cells with $CD44^{hi}CD122^+$ memory features have been described both in mice and humans (32, 33). These can arise in the thymus (innate-like memory cells), or in response to lymphopenia (virtual-memory T cell), and can contribute to protective anti-tumor immunity (32–35).

Overall, the heterogeneity of T_M subsets, with defined phenotypes, functions, and anatomical distribution, contributes to effective protective immunity. This has to be taken into consideration when developing ACT-based strategies, as adoptively transferred T cell products should closely mimic the behaviors of naturally occurring cells and be endowed with both effector functions, to promote acute tumor debulking, and long-term persistence, to promote surveillance against recurrent disease.

THE MAKING OF T CELL MEMORY: A THREE-SIGNAL BUSINESS

The strength and duration of TCR engagement by cognate peptide–MHC complexes (signal 1), co-stimulation (signal 2), and inflammatory cytokines (signal 3) contribute to naïve T cell priming and T_M differentiation (36–38). CD4 T_N cells require persistent TCR-peptide/MHCII interactions to achieve maximal clonal expansion (39). This can be regulated by the strength of TCR-peptide/MHCII binding (40) or by repeated contacts with Ag-bearing APCs, (41) and have direct consequences on T_{EFF} cell function (42). In contrast, CD8 T_N cells can be "programmed"

by short-term access to Ag to allow T_{EFF} differentiation, and prolonged and stable interaction with Ag-bearing APC appear necessary for full T cell activation and memory generation (43). Costimulatory ligand/receptor pairs, able to control the magnitude of the T cell response and the rate of T_M development and maintenance, generally provide signal 2. CD28 and members of the tumor necrosis factor receptor (TNFR) family, such as CD27, 4-1BB, and OX-40, in particular impact the formation and/or responsiveness of the memory CD4 and CD8 T cell pool [reviewed in (44)]. The expression of costimulatory receptor can be constitutive, as in the case of CD28 or CD27, or also inducible, as in the case of OX40 or 4-1BB, in response to IL-7 and IL-15, which promote T_M survival and homeostatic turnover (44). The importance of costimulation in adopting the appropriate T_M feature has also been demonstrated in ACT (45). For instance, exogenous agonistic anti-4-1BB IgG4 significantly promoted the yield of TIL expansion, and programmed them for enhanced survival and effector functions (36). Similar CAR-T cell engineering has evolved to include costimulatory domains in the original CAR construct. Initial studies demonstrated the beneficial effect of including the intracellular domain of CD28 to elicit both TCR and CD28 activation. Later, it became clear that a range of other costimulatory domains, including ICOS, and the TNFR superfamily members 4−1BB, OX40, and CD27, could rather promote long-lived memory cells (46). In addition, the engineering of defined CD28- or 41BB-costimulatory moieties within CAR constructs was proven to favor glycolysis (CD28) or mitochondrial biogenesis and oxidative metabolism (41BB) with direct implication for memory development *in vivo* (47).

Inflammatory cytokines (signal 3) also contribute to T cell priming. They do so by promoting T cell proliferation, effector functions acquisition, and long-term maintenance of protective immunity. At least three candidate cytokines, IL-12, type-I IFNs, and IFN-γ, have been shown to differentially contribute to CD8 T_M cell differentiation (7, 48). In the case of CD4 T cells, according to the type of immunological insult, the host-pathogen interaction, and resulting pro-inflammatory cytokine expression, promote differentiation of various T helper subsets (T_H1, T_H2, T_H17, T follicular helper cells/T_{FH}, T regulatory cells/reg) with pleiotropic effector functions, some degree of plasticity, and various memory-forming potential (31, 49, 50).

Overall, the characterization of the signals dictating T cell differentiation has been instrumental to identify specific manufacturing conditions for ACT. The use of TCR and costimulatory receptor engaging ligands, in combination with common γ chain (γc) cytokines and defined nutrients, can instruct T cells to adopt different T_M features, with direct consequences on T cell engraftment and long-term survival.

NOT ALL MEMORIES ARE GOOD ONES: THE CASE OF CHRONIC INFECTION AND CANCER

Any interferences in the three-signal model of T cell activation can result in dysfunctional phenotypes, sometimes endowed with inhibitory functions (51). For instance, defective co-stimulation, negative co-stimulation by inhibitory receptors, or anti-inflammatory cytokines can make T cells anergic and tolerant to cognate Ag, (52, 53) or induce the differentiation of T_{reg} cells. Ag-experienced tolerant T cells, compared with T_M, express high levels of co-inhibitory receptors (e.g., PD1, CTLA-4, TIM3, and LAG3) and transcriptional repressor (e.g., EGR1/2, DUSP2), low levels of cytokines and chemokine receptors, and mostly lack effector functions (53). In addition, continuous stimulation through the TCR, typically induced by Ag persistence during chronic infections or cancer, drives CD4 and CD8 T_M into a state referred to as T cell exhaustion (T_{EX} cells) (54, 55). T_{EX} cells are characterized by progressive loss of effector functions, metabolic deregulation, poor memory recall, and homeostatic self-renewal (54, 56). They acquire high and sustained expression of different inhibitory receptors, which are not found on T_M cells arising after resolution of an acute infection (57). Although CD8 T cell exhaustion was first described in LCMV chronic infection, (58, 59) it is now clear that it occurs in several other chronic infections, (60–62) in autoimmune disorders (63, 64) as well as in cancer (56, 65). T_{EX} cells, along with classical anergic T cells and T_{reg} emerging from the thymus or generated by the conversion of T_{EFF} cells, (66) might induce a dysfunctional state in tumor-infiltrating CTLs (67) and represent barriers to engraftment and function of ACT products. Accordingly, depleting strategies have improved responses to immunotherapy (68).

Although initially described in the context of infectious diseases, tumor-associated T_{RM} should also be carefully considered as their role in oncology has now been established (22, 69). In general terms, T_{RM} produce effector molecules such as IFN-γ, TNF-α, and IL-2 more rapidly than other memory T cells, (70) orchestrating the recruitment of auxiliary immune cells to the infected site (71). In tumor immunity, T_{RM} have nevertheless been reported to play controversial functions. For instance, CD8 T_{RM} cells driven by autoimmune vitiligo or primed by active vaccination conferred protection against melanomas, and their intra-tumoral representation was correlated with favorable prognosis in a variety of cancers (72, 73). In other studies, instead, tumor-associated $CD103^+$ CD8 T_{RM} showed regulatory properties (CTLA-4 and IL-10 expression), and were found to adopt a dysfunctional phenotype over time by expressing the highest levels of PD-1, TIM-3, CTLA-4, and LAG-3 (74). It should, however, be noted that tumor-associated T_{RM} can respond to anti-PD-1 treatment and were described to exert potent cytotoxic and effector functions in melanoma patients (75). Likewise, tumor-associated $CD103^+$CD4 T_{RM} have been described to be highly enriched for tumor-specific T cells (76) and suppress tumor growth through the secretion of TNF-α and IFN-γ or direct killing of tumor cells (77, 78). Again, these cells express high levels of co-inhibitory receptors, and yet whenever activated in appropriate conditions (such as by providing agonistic stimulation of CD27 or CD28 co-stimulatory molecules and/or immune checkpoint blockers) (79) might represent valuable allies and support tumor rejection. Otherwise, T_{RM} cells may be an obstacle to ACT products, as they might compete for local resources. For instance, T_{RM} cells are highly sensitive to IL-15,

a cytokine which newly transferred T cells depend on (80). Thus, a better understanding of the molecular mechanisms mediating CD4 and CD8 T_{RM} differentiation and interplay will allow harnessing the protective capacity of these memory subsets and modulate their activity in the context of ACT. In this respect, tumor-resident CD4 T_{RM} might reveal useful to provide local help to CD8 ACT products, and by that their function and survival.

ACT FOR TUMOR THERAPY: KNOCK-KNOCK, MAY WE COME IN?

Adoptive T cell therapy for tumor therapy is generally provided in the context of allogeneic or autologous settings. In the case of HLA-matched allogeneic donors, mature T cells comprise undefined T_N and T_M populations transferred at the time of stem cell transplant or shortly after (81–83). According to their nature and T_M composition, allogeneic T cells can provide graft-versus-tumor effects and also graft-versus-host disease owing to the presence of T cell reactive to minor histocompatibility antigens (84). In the autologous settings, instead, T cell products can be generated by the expansion of tumor-reactive cells isolated from tumor specimens (TILs) or by the genetic engineering of peripheral blood T cells with TCR or CAR, an antibody-derived single-chain variable fragment fused to T cell signaling domain(s), specific for tumor-specific/associated Ags (46, 85, 86). The phenotype of ACT T cell products can impact on therapy efficacy (87). Given that natural and manufactured T_M share similar requirements, they might compete for space, nutrients, cytokines, or TCR-engaging ligands *in vivo* (**Figure 1**). According to manufacturing culture conditions, T cells can acquire various T_M features. Which T_M cell subset represents the most effective in driving durable cures in cancer patients has been debated and remains to be fully elucidated and might vary according to the cancer type/state. Preclinical and clinical studies have shown that less differentiated T_{SCM} and T_{CM} display better expansion, persistence, and antitumor activity *in vivo* when compared with fully differentiated T_{EFF} (88–90). Accordingly, in retrospective analysis of ACT trials, more favorable objective clinical responses were found with less differentiated T cell products (88, 91). Although initial studies adopted IL-2 to support the *in vitro* expansion of engineered T cells, it soon became evident that T cell products had limited survival potential when transferred *in vivo* (92). Rather, shorter expansion times, and the use of IL-7, IL-15, and IL-21 provided T cells with longer persistence *in vivo* (92). As T_{SCM} and T_{CM} are found in limited number in the peripheral blood and at the tumor site, *in vitro* methods have been defined to generate them starting from T_N precursors. These include polyclonal activation (αCD3/28 antibody—conjugated beads), homeostatic cytokines (IL-7, IL-15, and IL-21), (93–95) inhibitors of specific signaling pathways (such as GSK3β, AKT, or mTOR) (96, 97) or epigenetic regulators, (98) and nutrients/metabolites aimed at arresting terminal differentiation and promoting memory stem cell phenotype, during *ex vivo* T cell expansion. The same extracellular cues that guide the manufacturing tumor-reactive lymphocyte continue to affect the activity of adoptively transferred T cells, which, as introduced in

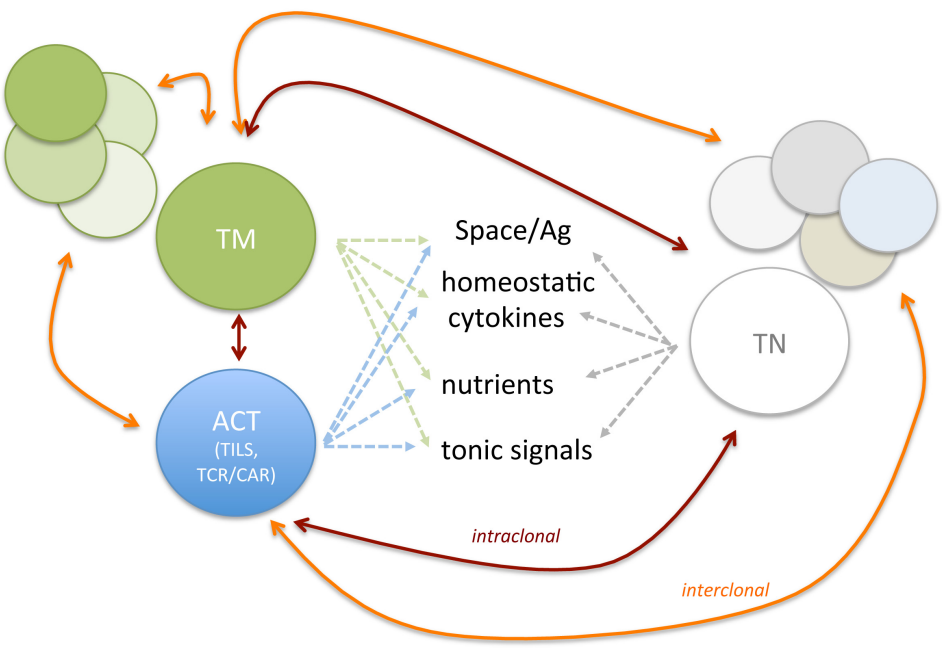

FIGURE 1 | Endogenous and ACT T cell subsets compete for space and resources. Are new comers strong enough to outcompete long-term resident? The figure depicts possible competition among T_N, Ag-experienced T cells (T_{CM}, T_{EM}, T_{EFF}, T_{RM}, T_{EX}, and T_{reg}), and ACT. Both intra-clonal competition (among T cell subsets expressing the same TCR, red lines) and inter-clonal competition (among T cell subsets with different TCR, orange lines) might shape persistence of newly infused T cells and the composition of endogenous T cell repertoire.

the previous section, compete with pre-existing T_M subsets and the tumor microenvironment (TME), once re-infused in patients. We argue that this competition might have direct consequences on *in vivo* differentiation and survival of ACT products and their therapeutic effect (**Figure 2**).

CELL INTRINSIC AND CELL EXTRINSIC EVENTS WITH THE POTENTIAL TO SHAPE THE HOST–ACT T_M CROSS-TALK

Both cell-intrinsic and cell-extrinsic events shape T_M differentiation, survival, and functions. In this paragraph, we review some of the available evidence that support the impact of clonal abundance, TCR affinity, and availability of cytokines and nutrients on T_M behavior and their possible implication in the host–ACT T_M cross-talk. As TILs and TCR-engineered T cells recognize MHC restricted antigens, while CAR-T cells bind surface antigens via MHC-independent mechanisms, the impact of such critical determinants might vary and be relative to each given ACT product.

Clonal Abundance

In natural immunity, initial clonal abundance, i.e., the frequency at which any given TCR is represented within the polyclonal repertoire, and the relative TCR affinity for cognate antigens have been shown to give rise to intraclonal and interclonal competition and shape the T_M repertoire [recently discussed in (99)]. Data have shown that natural differences in the size of foreign Ag-specific T cell populations have direct consequences on the magnitude of the effector and memory response (100, 101). Intraclonal competition has been shown to affect the expansion and accumulation of both T_N and T_M naïve cells and to block the proliferation of adoptively transferred CD8 T cells (102). In cases where T cells exceed the relatively narrow physiologic range, intraclonal competition was observed with consequences on overall T_M survival and expansion potential (103). Among several factors, competition for Ag and/or access to Ag-bearing APC played a role, (104–106) which caused suboptimal T cell expansion, and defective generation of effector and memory T cells against foreign pathogen and tumors (107–109). In the case of CD8 T cells specific for a tumor-associated self-Ag, increasing naïve T cell frequency at supra-physiological

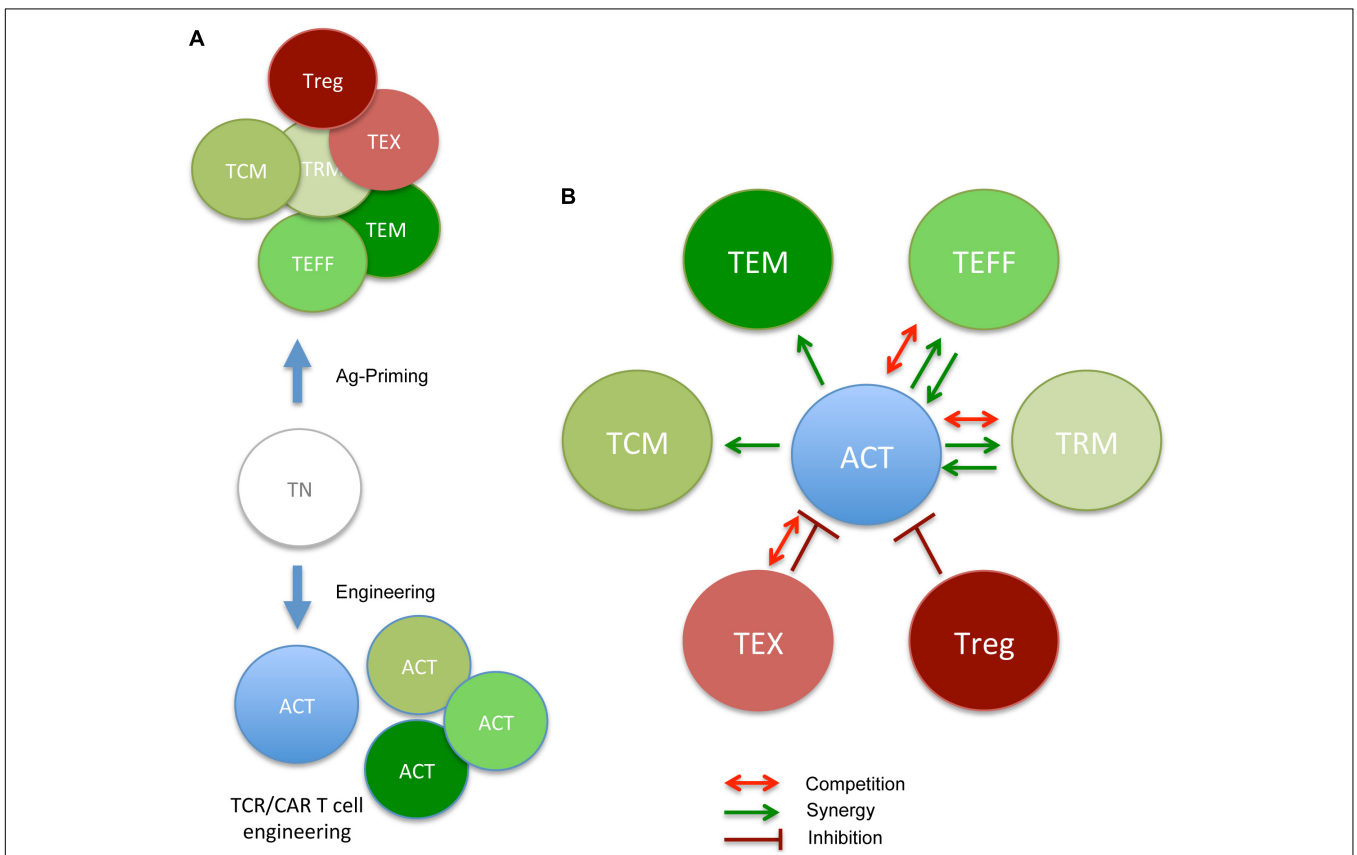

FIGURE 2 | ACT-derived T cell products and cross-talk with pre-existing memory/Ag-experienced subsets (the good, the bad, and the ugly neighbors). **(A)** During *in vivo* Ag priming or *in vitro* engineering/expansion, T_N acquire diverse phenotypes, reflecting various degrees of differentiation (T_{CM}, T_{EM}, T_{EFF}, T_{RM}, T_{EX}, and T_{reg}). Each subset reveals defined requirements for proper effector function and survival. **(B)** In the context of ACT, newly infused T cells will interact with pre-existing subsets, and either benefit from good memory subsets or take advantage of them (T_{CM}, T_{EM}, T_{EFF}, and T_{RM}) in promoting therapeutic anti-tumor responses (green arrows) or be inhibited by nasty ones (T_{EX} and T_{reg}). In some cases, competing for nutrients/survival signal (**Figure 1**) might also dim ACT efficacy (red arrows). Putative collaborative and antagonistic relationship between pre-existing memory subsets and ACT cell products are depicted.

precursor frequencies was reported to ameliorate responses to tumor-specific vaccination and therapeutic effects. Nevertheless, above a certain threshold, intraclonal competition was observed, and this limited protective immunity (110). We also found, in a model of spontaneous prostate cancer development, that increasing the number of TCR-transduced T cells or repeating their administration over time did not significantly increase the relative abundance nor their therapeutic potential (111). Of note, in some models, sequential administration of tumor-specific CTL proved more efficacious than the single initial injection of an equivalent CTL number (112). Thus, data support the notion that intraclonal competition regulates both naturally occurring and ACT-derived antigen-specific CD8 T_M.

Intraclonal competition was also reported to regulate relative abundance of T_N and T_M CD4 T cells (and in the case of tumor/self Ag-specific naïve CD4 T cells). By transferring different numbers of tumor-associated self-Ag-specific TCR transgenic CD4 T cells, Malandro and co-authors showed that initial precursor frequency inversely correlated with *in vivo* expansion and functional outcomes. Although at low precursor frequency CD4 T cells specific for a tumor-associated self-Ag underwent robust proliferation, they acquired an irreversible exhausted phenotype (113). In contrast, at higher precursor frequencies, T cells showed a poor expansion potential, but preserved an optimal cytokine-secretion profile and antitumor activity. Interestingly, the authors showed that above a certain threshold, intraclonal CD4–CD4 T cell help cooperation became evident (113). It is tempting to speculate that increasing the frequency of tumor-specific CD4 T helper cells might provide a selective advantage to both naturally occurring and ACT-derived T_M. Whether increasing the availability of CD4 T cell help might be beneficial to ACT approaches, and whether predefined CD4 and/or CD8 T cell compositions at various degree of differentiation would ameliorate ACT therapeutic potential is currently being investigated in a number of ongoing trials (91, 114).

TCR Affinity/Avidity

Availability of the cognate antigen or access to cross-reactive self-Ag appears to be a critical determinant in dictating clonal abundance within a memory subset and/or the emergence of high/low affinity TCRs. In the case of CD4 T cells, weak TCR signals from self-peptide MHCII ligands are important for T_M survival, and this becomes limiting in the presence of high frequencies of CD4 T cells specific for the same ligand (103, 115). In the case of CD8 cells, this is critical for shaping the T_M repertoire, but less so for T_M maintenance, which predominantly depends on cytokines such as IL-15 and IL-7 (116, 117). Nevertheless, TCR affinity/avidity contributes to clonal selection. It regulates access to vital co-stimulatory molecules, cytokines, and nutrients, (99) and it determines that clonotypes with higher affinity (and slower TCR–ligand dissociation rates) acutely increase in frequencies within T_M pools, above levels reached during the course of anti-viral immunity starting from naïve precursors (118, 119). During acute murine cytomegalovirus (CMV) infection, for instance, a subset of high-avidity virus-specific CD8 T cells typically increases in size (clonal dominance) and simultaneously establishes a large pool of effector memory T cells able to outcompete lower avidity CD8 T cells (120, 121). This mechanism has now been defined as "memory inflation" and documented over the course of several viral infections (122, 123). In a recent study, Schober and colleagues studied TCR repertoire evolution in the context of latent CMV infection and found that, although high-affinity TCRs dominated T cell responses at early times after infection, low affinity TCRs emerged over time, owing to cellular differentiation and senescence (and not exhaustion) of high affinity ones (124). In a recent publication, Poschke and coauthors exploited TCR deep sequencing to characterize TILs before and after *in vitro* culture and found that dominant T cell clones were lost during TIL culture because of poor expansion potential, in favor of less represented ones (125). The authors argue that spatial heterogeneity of the tumor T cell repertoire, as well as differences in intrinsic *in vitro* growth capacity between individual T cell clones, influenced the T cell preparation. In melanoma, the most abundant T cell clones were found to be tumor reactive, and yet, neo-antigen-reactive T cells, of possible highest affinity, were gradually lost during TIL expansion. Whether this occurs also over the course of natural evolution of anti-tumor immunity and/or under the pressure of ACT remains to be determined. Yet, it is tempting to speculate that in some cases a switch in dominance toward low-affinity TCR T cells might indeed take place during the editing process, especially under the pressure of high-affinity ACT T cells and precede and/or account for final tumor escape.

The remodeling of the host T cell repertoire was observed in ACT for both mouse and human CMV. In mice, adoptively transferred T cells were shown to restrict the repertoire of host-derived T cells via competitive mechanisms, supporting clonal dominance of T_M ACT cells over endogenous memory cells (126). In the setting of anti-CMV-specific ACT, T_M influenced further responses by endogenous CMV-specific T cells in organ transplant recipients (127). This was best observed in patients who had an unbiased TCR repertoire before the transplant, and correlated with therapy efficacy, likely owing to further expansion of viral-specific T cells. Instead, non-responding recipients revealed a pre-transplant biased peripheral T cell repertoire, which was not influenced by ACT. This indicates the ability of ACT to promote a restructuring of the T cell pool, given proper immune cell representation pre-ACT. Because adoptively transferred T cells synergize with endogenous responses (128) (discussed in following paragraph), future studies would be needed to understand how TCR/CAR-T cell impact on the representation of pre-existing and newly generated tumor-reactive T_M cells.

Thus, when considering ACT, a detailed characterization of host immune competence might help predict efficacy, and also instruct optimal T cell product composition. Of note, CAR-T cells might be expected to be less sensitive to the competition with endogenous T_M subsets than TILs or TCR redirected T cells, at least for TCR engaging ligands and deriving tonic stimulation. In the case of CAR-T cells, antigen-independent tonic signaling has been shown to result from spontaneous clustering of CAR molecules. However, in contrast to tonic TCR signaling, this event was associated with augmented T cell apoptosis, exhaustion,

and impaired antitumor effects (129, 130). As preclinical studies suggest that CAR–TCR interactions are a prerequisite for optimal CAR-driven T cell activation, (131) it remains possible that survival of CAR-T_M cells is controlled by the same signals that support TCR T cells. If this would be the case, then also CAR-T cell might be sensitive to surrounding endogenous T_M subsets.

Cytokines

In the context of natural immunity, competition for γ_c cytokines, principally IL-7 and IL-15, regulates the balance of CD4 and CD8 T_N and T_M cells, homeostatic proliferation, and survival (132–134). Only those T cells receiving sufficient signaling escape the apoptotic process and proliferate (135–137). IL-7 mostly supports CD4 and CD8 T_M, whereas IL-15 also promotes their homeostatic proliferation. Given the higher expression of IL-2Rβ, CD8 T_M cells are more sensitive to IL-15, and outcompete CD4 T_M and T_N cell subsets during acute infections and homeostatic proliferation (138, 139).

T cells transferred in the setting of ACT are also sensitive to γ_c-cytokine availability. Accordingly, cytokines like IL-2, IL-7, IL-15, and IL-21 are fundamental both for generation of the ACT cell products and to increase the efficacy and the duration of the anti-tumor response *in vivo*. Administration of low-dose IL-2 to the patient after ACT therapy has generally been used to enhance the *in vivo* persistence of the newly adoptively transferred T cells which has been shown to translate in a favorable clinical outcome (140). Although initial studies adopted IL-2 to support the *in vitro* expansion of engineered T cells, it soon became evident that T cell product had limited survival when transferred *in vivo* (92). Rather, shorter expansion times and the use of IL-7 and IL-15 mediated the selective expansion of CD4 and CD8 T cells, while limiting the representation of T_{reg} cells, resulting in longer persistence *in vivo* (141–143). Because of their capacity to support, both *in vivo* and *ex vivo*, T_M cell generation and homeostatic proliferation, IL-7 and IL-15 are now being evaluated in human clinical trials. IL-21 also plays an important role in ACT manufacturing based on its ability to significantly enhance the *ex vivo* generation and TCR affinity of T_M cells (144, 145).

As in the case of TCR engaging ligands, endogenous and ACT T_M might compete for cytokine availability. Thus, once more the T cell repertoire before ACT might influence ACT efficacy, and vice versa recently infused T cells could impact on endogenous subsets. Accordingly, lymphodepletion by various pre-conditioning protocols has been shown to promote both the engraftment and the long-term persistence of TILs, TCR, (142, 146, 147) and also of CAR-T cells (148–150) by lowering the number of immune cell subsets, which compete with transferred tumor-reactive T_M for the γ_c-cytokine binding (142). Clinical trials in melanoma patients first indicated that TIL persistence was improved by preconditioning of the patients with lymphodepleting strategies based on total body irradiation (151, 152). Preclinical models further demonstrated that lymphodepleting regimens improve engraftment and functionality of transferred T cells prolonging their survival, via IL-15-dependent signaling (153). The competition for homeostatic cytokines might also evolve in a "metabolic competition" that might render ACT T_M dysfunctional and metabolically exhausted. Indeed, IL-15 also regulates T_M metabolic stability (154). In this respect, data indicate that IL-7 administration following cyclophosphamide preconditioning supports the expansion of polyfunctional tumor-specific CD4 T cells (155). Thus, in this scenario, we speculate that lymphodepletion is able to contribute to effective anti-tumor immunity because, in addition to be critical to eliminate immune-suppressive cells (i.e., MDSCs, TAM, T_{EX}, and T_{regs}) and decrease the metabolic competition in the TME for IL-7, IL-15, and nutrients, it bears the potential to support both ACT T_M and possibly also endogenous T_N and T_M.

Nutrients

Nutrient availability is a requisite for proper T_M function and long-term persistence. T_M cells own a unique metabolic signature. On Ag encounter, T cells engage OXPHOS, glutaminolysis, and glycolysis to fulfill bioenergetic and biosynthetic demands needed to support proliferation and effector functions. After Ag clearance, T_{EFF} cells reduce their metabolic demands and dependence on glycolysis, and gradually reset back from an anabolic to a catabolic state, typical of long-lived T_M cells. At difference with T_{EFF} cells, T_{CM} cells are mostly quiescent and adopt a metabolic profile similar to T_N cells, whereby they rely on mitochondrial metabolism and fatty acid oxidation to support their persistence and functions (156, 157). T_M cells also possess greater mitochondrial mass and enhanced Spare Respiratory Capacity, (158) two key metabolic features important not only for their development and long-term survival but also for their rapid recall ability.

In the context of ACT, metabolic fitness plays an important role. T cells with high metabolic activity and glycolytic rate are endowed with potent T_{EFF} functions (i.e., capable of cytokine production and rapid proliferation). The identification of the metabolic pathways critical to T_M survival and functions supported the concept that *in vitro* metabolic reprogramming could impact *in vivo* tumor activity. As a consequence, several approaches targeting T cell metabolism *in vitro* and *in vivo* by targeted delivery of metabolism-modulating compounds to the TME have been investigated for effective cancer immunotherapy and recently reviewed (93, 94).

Nevertheless, within the TME, tumor cells and/or other infiltrating immune subsets share metabolic requirements, which are overlapping with those of ACT products. Tumor cells indeed frequently share a similar glycolytic metabolic profile with activated T cells, and compete with them for fundamental nutrients, hindering the ability of effector T cells to meet energetic needs. Two studies showed that highly glycolytic tumors can deplete glucose levels in the TME, dampening the ability of T cells to maintain anabolic growth signaling and produce inflammatory cytokines (159, 160). Moreover, by consuming glucose and producing cAMP, tumors limit T_{EFF} functions and instead promote T cell senescence (161). Additional studies highlight that high rates of amino acid uptake by tumor cells could potentially inhibit the anti-tumor T cell response because amino acids are crucial nutrients that support T cell proliferation and effector functions (162, 163). There is also

emerging evidence that some tumors uptake fatty acids with high rates to maintain their proliferation (164). Given that fatty acids have a central role in T cell memory differentiation and function, (165) lipids may be another nutrient that T cells must compete for in the TME.

In the context of this competition, it is worth noticing that both CD8 T_{RM} and T_{CM} cells depend on mitochondrial FAO and OXPHOS. However, to fuel this metabolic pathway, CD8 T_{RM} cells strictly depend on uptake of exogenous fatty acids, (166) whereas CD8 T_{CM} cells rather use extracellular glucose to fuel this process. Thus, CD8 T_{RM} cells might be engaged in a metabolic competition with adoptively transferred CD8 T_{CM} cells to fuel mitochondrial metabolism. Whether such competition for nutrient uptake impacts on ACT product functionality remains to be investigated.

The type of nutrients available to T cells, such as glucose, lipids, and amino acid, influences the differentiation program, functional properties, and shape their ability to control tumor progression (167). Although T cell metabolic reprogramming to a given metabolic fitness might be achieved during manufacturing, it should be considered that programming T cells *in vitro* might have a detrimental outcome *in vivo*, as the tumor itself, stromal cells, and/or other infiltrating immune cells compete for critical nutrients. For instance, T cells addicted to glycolysis during *in vitro* culture might experience nutrient deprivation when transplanted in the host and die because of insufficient glucose availability. Therefore, to mount an effective anti-tumor response, ACT products should retain the metabolic flexibility to adjust to nutrient availability. In this scenario, insights into the metabolic characterization of the TME might help inform *in vitro* metabolic re-programming of tumor-specific T cells to ameliorate their persistence and long-term survival in a metabolic unfavorable TME.

TO REMEMBER OR TO FORGET: GOOD, BAD, AND UGLY MEMORIES

Pre-clinical and clinical studies have shown that pre-existing memory T cells contribute to the efficacy of immunotherapy and radio-immunotherapy (168–171). Several evidences indicate that also in the case of ACT with TCR/CAR engineered products, host T cells contribute to the therapeutic anti-tumor responses. The heterogeneity within the responding pool of T_M, owing to the ability of the cells to integrate several signals (TCR, co-stimulatory molecules, and cytokines), their differentiation status, and their relative fitness in an environment of rapidly expanding cells competing for the same resources, is thus likely to impact on the final therapeutic outcome.

Could then pre-existing memory or on a more general definition, Ag-experienced T cell subsets be stratified according to their impact on ACT efficacy? We speculate that this could be the case, in relation to their relative function in protective responses, as they could improve or hinder ACT products. "Good memories" might be represented by those subsets of cells, endowed with effector capabilities and able to synergize with tumor-specific T cells provided by ACT. For instance, pre-existing T_M or T_{RM} might respond to pro-inflammatory signals generated in response to intra-tumoral activation of TCR/CAR engineered T cells, (172) and contribute to anti-tumor immunity by conditioning the immune milieu and/or exerting direct anti-tumor activity. In a recent report, Walsh et al. showed that adoptively transferred T cells synergized with endogenous T cells, which were instrumental to prevent immune escape of Ag-loss variants. Post-transplant tumor-specific vaccination supported better tumor infiltration and prolonged survival of tumor-reactive lymphocytes, likely promoting intra-tumoral responses and Ag cross-presentation (128). Local activation of T_{RM} cells might in turn favor the spreading of circulating CTL responses against tumor-derived neo- and self-antigens (173). We also found that tumor-specific vaccination promoted optimal anti-tumor immunity when applied after allogeneic hematopoietic cell transplantation or ACT with tumor-redirected TCR engineered T cells. Also in these settings, in addition to engineered T cells, non-transduced cell subsets were revealed capable of IFN-γ and Granzyme-B expression (174–177). Similarly, Ma and co-authors found that the implementation of a vaccine boosting via the CAR (using a smart by-specific vaccinable CAR) enhanced CAR T cell expansion and also favored the recruitment of additional specificities (178). It is possible that infiltrating T cells, positively selected in the thymus by virtue of expressing TCRs with a low/intermediate affinity for self-Ags, are indeed able to recognize such antigens on tumor cells, or that TCR/CAR-induced inflammatory environment promotes effector function by bystander cells via non-Ag-specific mechanisms (179–181). In this respect, it is worth mentioning that robust and iterative stimulation of memory self-specific CD8 T cells reverted tolerance to self in the context of acute infection, and promoted anti-tumor immunity, without precipitating autoimmune manifestations (182). This is reminiscent of the synergy between T cells specific for a tumor and a Y chromosome–derived self-antigen in the context of allogeneic hematopoietic cell transplantation and in ACT with TCR-redirected T cells (174, 175). These studies suggest to exploit the use of pre-existing or newly infused T_M possibly reactive to self/tumor-associated Ags in ACT of tumors.

Viral-specific memory T cells could also come to help. It is known that both mouse and human tumors are commonly surveyed by memory T cells specific for previously encountered viral infections (183). A recently published manuscript showed that these functional T cells can be specifically reactivated via the local delivery of viral peptides, which caused a local inflammatory environment capable of activating both the innate and adaptive immunity, leading to tumor growth arrest. Immunization with viral peptides sensitized mice to PD-L1 checkpoint blockade promoting the elimination of otherwise resistant tumors (169). Thus, viral-specific memory T cells, if appropriately activated, might synergize with ACT.

Finally, resident T_{RM}, bystander memory subsets, and virtual memory T cells might also play a role, as capable of responding to pro-inflammatory cytokines, known to lower the threshold for T cell activation and/or induce TCR-independent effector functions (180, 184). Supporting this, CAR T cells engineered to express IL-12 and/or IL-18 (TRUCK T cells) have proven more effective than those only expressing the tumor-specific CAR (185, 186).

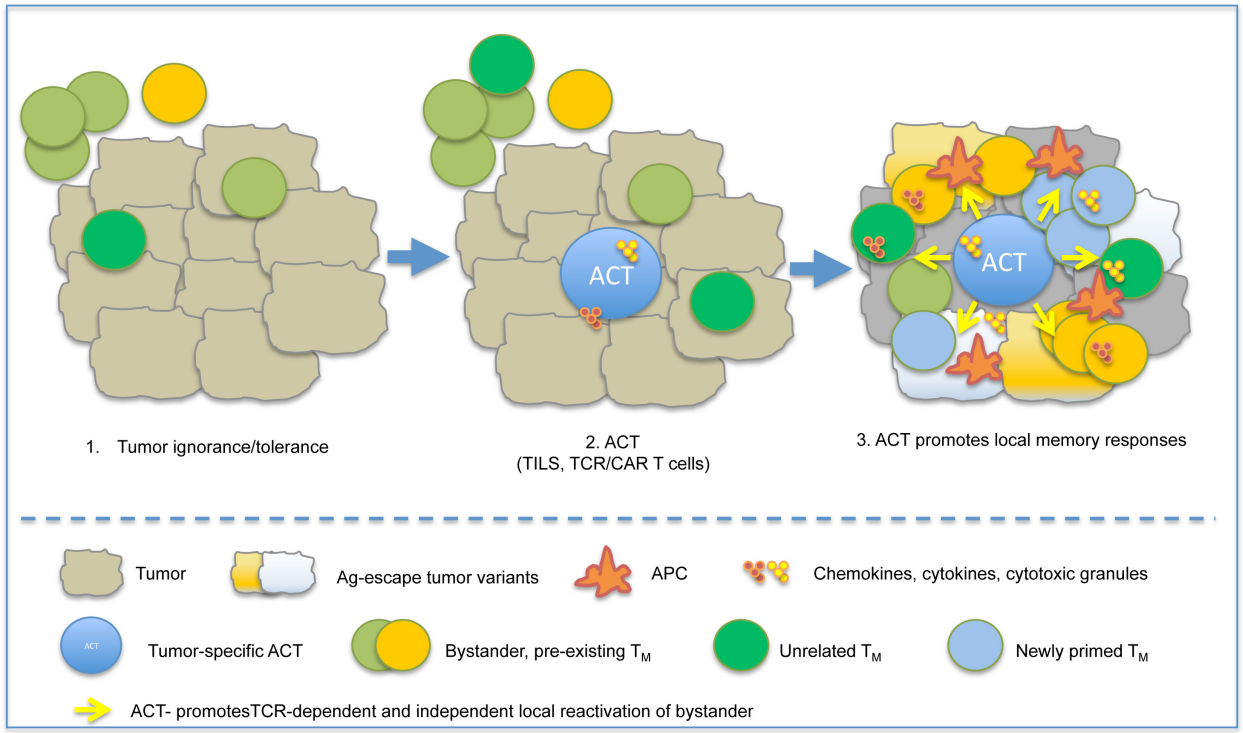

FIGURE 3 | ACT instructs protective immune responses in tumors. A schematic model depicting the possible influence of ACT on pre-existing memory is depicted. Shortly after infusion, ACT-derived T_{EFF} recognize tumor antigen, cause tumor cell death and release of pro-inflammatory cytokines (IFN-γ, TNF-α), and promote local inflammation. This causes recruitment of APC and bystander memory subsets, and their local reactivation by either TCR-dependent or TCR-independent mechanisms.

TCR could cross-recognize multiple Ags or respond to unrelated Ags, self-Ag, or environmental Ags (187, 188). Virtual memory T cells can produce IFN-γ and are capable of Ag-independent lytic activity, in response to the inflammatory milieu alone, i.e., when stimulated with IL-12, IL-15, and IL-18 (189).

Although in several instances the cellular and molecular mechanisms at the base of the synergistic effect need further investigation, bystander T memory cells were frequently found within the tumor infiltrates, (180) suggesting that pre-existing memory cells, unrelated to the cognate tumor-associated Ag targeted by TILs or TCR/CAR T cells, might contribute to anti-tumor immunity in ACT settings (**Figure 3**).

Along this line, it should be mentioned that unfortunately, also "bad memories" exist, which may impair the development of new memories. This was shown to be the case in the well-recognized phenomenon of "original antigenic sin," where an existing immune response prevents the initiation of a later, cross-reactive but independent immune response. This phenomenon, which was described for antiviral CD8 T cell immune responses (190, 191) and in vaccinated mice, owing to the ability of pre-existing effector cells to eliminate Ag-bearing dendritic cells, might play a role in limiting propagation of anti-tumor protective memory responses. Accordingly, we found that anti-tumor CD4 T cell responses limited efficacy of active vaccination in tumor-bearing mice, (192) supporting the possibility that in some cases pre-existing memory might counteract a newly born one.

Although in real life it is well conceivable that these "bad memories" might protect individuals from recurrent infection/disease, in the context of tumor immunity, they might represent an obstacle to therapeutic efficacy.

Finally, even if anergic and T_{EX} cells, putative representative of bad memory T cell subsets, might appear to have no apparent function, they might consume useful resources needed for the efficacy of ACT cell products. This has been shown for T_{reg} subsets, which, for instance, can induce T_{EFF} cell senescence by competing for glucose and inducing DNA damage (193). Hence, this subset might prove to be an "ugly neighbor," capable of providing active suppression and inhibiting tumor recognition by ACT T cell products right from the start.

CONCLUSION

Given the aforementioned evidence, should pre-existent memory be considered before adoptive T therapy? The authors believe that it should.

The degree of heterogeneity within a T cell pool depends on the integration of signals from the TCR, co-stimulatory molecules, cytokines and nutrients, and also on the relative cell fitness within an environment competing for the same resources. Newly infused T cells would need to face and adapt to such pre-existing conditions, to engraft and exert proper

anti-tumor activity. The concept of immunological memory foresees that pre-existing memory T cells would be beneficial for protection against reinfection with the same pathogens, because Ag-specific memory T cells would be numerically increased when compared to endogenous ones, have widened anatomical distribution, and respond more quickly, conferring rapid clearance of the infectious agent. Nevertheless, previous encounters with the Ag has the potential to generate in addition to good memories (T_{CM}, T_{EFF}, and T_{RM}), also bad and ugly memories (T_{EX}, T_{reg}, and in some instances tumor-associated T_{RM}), with opposite effects on recall responses, and on the efficacy of adoptive T cell therapy. The quality and quantity of adoptively transferred cells are also important parameters to consider when optimizing such a treatment. "*The more is the better*" might not be the right choice, as cells should find sufficient space and support (TCR/CAR engaging ligands, homeostatic cytokines, nutrients) in spite of host–cell competition. Modeling tumor–immune system competition might help predict responses *in vivo* (112, 194). Would also provoking immunological amnesia promote therapeutic efficacy of T cell products in the context of ACT? We speculate this might not be entirely the case because as in real life, retaining positive memories might help. The finding that endogenous T cells cooperate with TCR/CAR-redirected T cells supports this statement (128, 195). Rather, strategies suitable to evoke selective amnesia from *bad* memories (i.e., capable of depleting/inhibiting regulatory subsets, and/or overcome competition for space or nutrients) would empower *good* ones and amplify therapeutic effects of current ACT products. As an alternative, synthetic biology and genetic engineering might help design T cell products insensitive to competition or able to metabolically adapt to hostile TME. Likewise, understanding and exploiting CD4 and CD8 T_M representation, both within the ACT product, and the pre-existing endogenous repertoire, might open new avenues of intervention. Thus, interesting challenges ahead will be to understand the cross-talk and homeostatic regulation between adoptively transferred T cells and endogenous ones to define strategies to eliminate any unneeded immunological bad memories, and take advantage of available local resources. This will foster productive synergies and supportive environments to render ACT products highly functional.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

AM acknowledges the support of Associazione Italiana per la Ricerca sul Cancro (AIRC: IG 2014-15883 and IG 2018-21763) and of TRANSCAN (TRS-2016-00000373). TM is the recipient of a Start Up Grant from Associazione Italiana per la Ricerca sul Cancro (AIRC StartUp 2018-21474).

ACKNOWLEDGMENTS

The authors wish to acknowledge the work of all those colleagues that we were not able to cite for space constraints.

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The handling editor declared a past co-authorship with one of the authors AM.

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