ON THE ORIGIN AND FUNCTION OF HUMAN NK-LIKE CD8+ T CELLS: CHARTING NEW TERRITORIES

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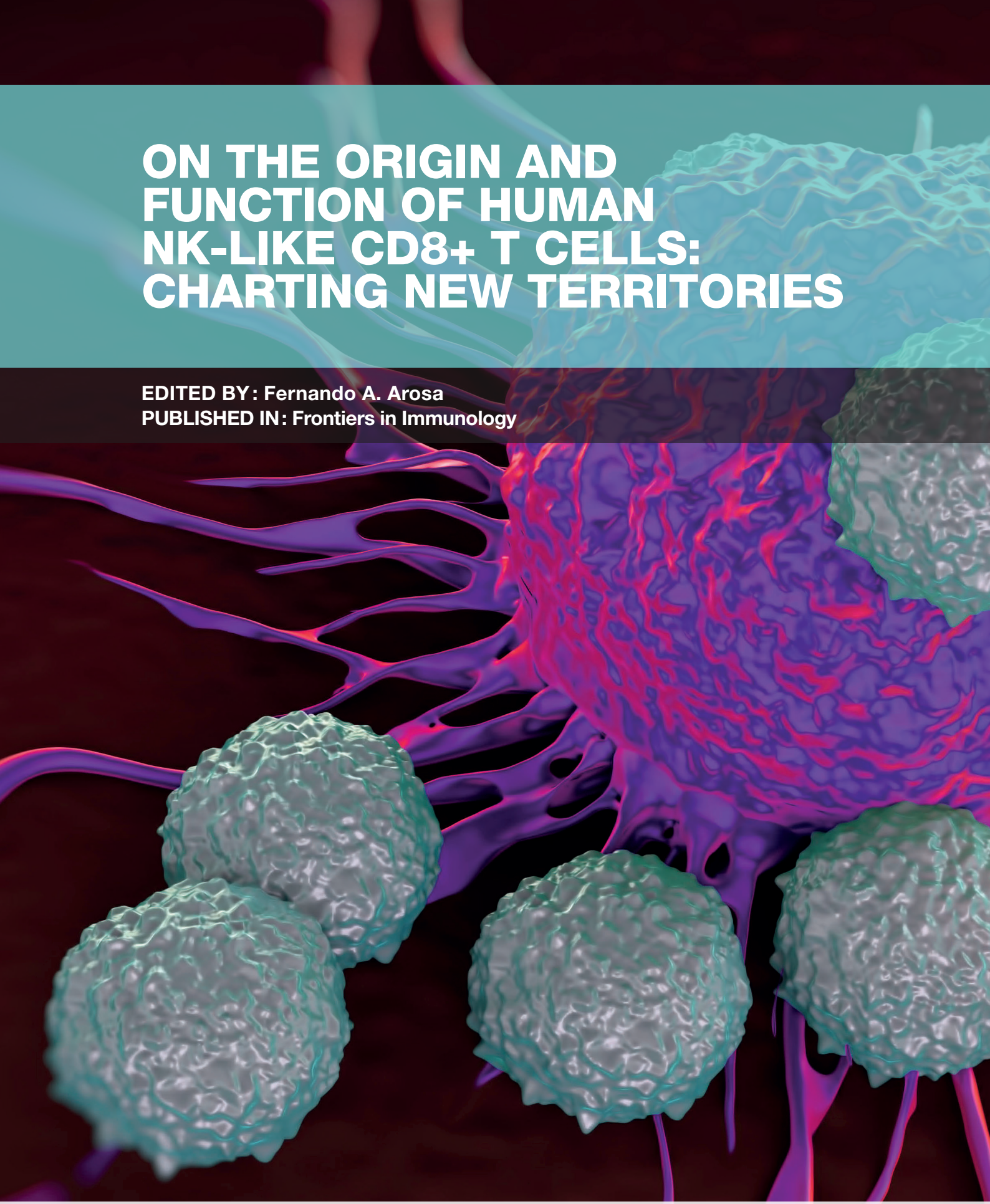

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ON THE ORIGIN AND FUNCTION OF HUMAN NK-LIKE CD8+ T CELLS: CHARTING NEW TERRITORIES

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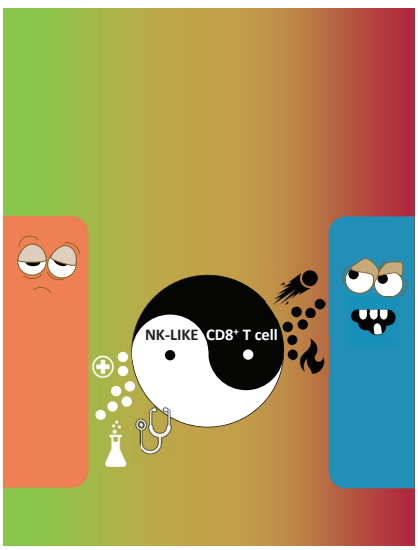

Cartoon illustrating the multifunctional role of human NK-like CD8+ T cells within peripheral tissues. Image: Elsa Cardoso.

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Human CD8+ T cells expressing NK receptors and receptors found on innate immune cells, and designated as NK-like or innate CD8+ T cells, have been long considered as terminally differentiated lymphocytes responsible for tissue inflammation and destruction. However, a growing body of knowledge is unveiling that NK-like CD8+ T cells have many, sometimes contrasting, functions. The limited knowledge of the biology of this type of CD8+ T cells and the role they play within peripheral tissues and organs under homeostatic conditions has hampered our understanding of disease and therefore the possible development of disease diagnostic tools and effective immunotherapies. In this Research Topic are presented a variety of topics and views, some of them overlooked for many years, on human NK-like CD8+ T cells, which may open new and novel avenues of research to further our understanding of these polyfunctional T cells.

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EDITORIAL
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Editorial: On the Origin and Function of Human NK-Like CD8+ T Cells: Charting New Territories

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Keywords: CD8+ T cells, suppressor, regulatory, aging, cancer, infection, inflammation, homeostasis

Editorial on the Research Topic

On the Origin and Function of Human NK-Like CD8+ T Cells: Charting New Territories

Human effector-memory CD8+ T cells displaying characteristics of NK/innate cells are a heterogeneous pool of multifunctional lymphocytes that are predominantly found in peripheral tissues and organs (1–3). In terms of phenotype, functional plasticity, and localization, NK-like CD8+ T cells are in a privileged place to regulate basic physiological processes, from immune protection against pathogens to wound healing and tissue regeneration, as shown for other T cell populations (4–6). The goal of this research topic was to bring novel insights into the origin and function of human NK-like CD8+ T cells.

The notion that NK-like CD8+ T cells share innate and adaptive features is elegantly described by Pereira and Akbar. In their review, they summarize the phenotypic, functional, and transcriptomic features that shape the generation of NK-like CD8+ T cells during human aging, including the signaling pathways involved. The authors propose that human NK-like CD8+ T cells are not dysfunctional, but a distinct T cell population that compensates for functional defects of conventional NK and CD8+ T cells. In line with this thinking, Michel et al. propose that the increase in NK-like CD8+ T cells seen in aged healthy people represents a remodeling of the T cell compartment to cope with physical and cognitive deleterious changes that take place with aging. In their review, they show an association between certain NK-like CD8+ T cell subsets and healthy aging, which led them to propose that there are subsets of NK-like CD8+ T cells that are bioindicators of successful aging and longevity. Although CMV seropositivity has long been considered a driving force behind the expansions of NK-like CD8+ T cells seen in the elderly, this view is changing. Thus, Saavedra et al. report data regarding the lack of association between CMV seropositivity and accumulation of NK-like CD8+ T cells in the elderly. By studying a cohort of CMV-infected young and elderly healthy Cubans, they found that age, but not CMV, was the main driving factor influencing the expansions of NK-like CD8+ T cells, which is in agreement with the studies referred by Michel et al. These data point to aging-related factors, among others, as responsible for the NK-like CD8+ T cell expansions, as discussed in the article of Pita-López et al. By performing a comprehensive analysis of the phenotypic characteristics, function, and development of human NK-like CD8+ T cells in the context of aging, autoimmunity, cancer, and infection, they conclude that the molecular cues responsible for the generation of NK-like CD8+ T cells as well as their exact function is still a matter of debate that warrants further investigations.

Although NK-like CD8+ T cells is a relatively recent designation, these cells were originally described as suppressor, as discussed in a more focused context by Xu et al. The authors engage in a comprehensive historical review of the phenotypic and functional features of human suppressor

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CD8+ T cells in the context of transplantation, autoimmune diseases, and viral infection, highlighting their CD28−, KIR+, CTLA-4+, PD-L1+, and Foxp3+ phenotype. They carefully review the mechanisms used by suppressor CD8+ T cells to inhibit T cell responses, highlighting the role of the inhibitory receptor ILT3 expressed by dendritic cells, and discuss novel immunosuppressive therapies. The origin and function of human NK-like CD8+ T cells were reviewed in a broader context by Arosa et al. In view of the expression of a highly diverse array of innate receptors, including receptors that trigger amphiregulin secretion such as IL-18R/IL-33R/ST2 (5), the authors envision NK-like CD8+ T cells as a highly experienced population with the skills and expertise to sense and cope with alterations that take place within an ever-changing environment, to keep tissues and organs intact and functional, in part by way of tissue regeneration. The authors also propose that open MHC class I conformers expressed by metabolically active cells, dividing cells, and stressed cells are important players that need to be taken into account to understand NK-like CD8+ T biology. In this regard, Cardoso and Arosa discuss the possible bone protective role of a subset of gingival NK-like CD8+ T cells during periodontitis. Based on recent data, the authors propose that gingival CD8+ T cells contain a pool of NK-like CD8+ T cells with regulatory/suppressor function, and these cells could be involved in the maintenance of alveolar bone integrity by constitutively downregulating inflammation under homeostatic conditions and initiating repairing mechanisms in case of tissue injury. The authors also acknowledge that under overt pathogenic bacterial colonization this protective role may be surpassed by the bacterial immune response and led to bone loss.

The surpassing of the proposed protective side of NK-like CD8+ T cells within inflamed tissues is addressed by Hodge and Hodge. The authors review recent data, including their own, pointing to populations of NK-like CD8+ T cells as involved in the inflammation of the airways in patients with chronic obstructive pulmonary disease. Importantly, the authors discuss recent data indicating that inflammatory NK-like CD8+ T cells are resistant to steroid treatment, reinforcing the need for novel therapeutic approaches. In line with this study, Lourenço et al. discuss the possible functional roles of NK-like CD8+ T cells in asthma. Based on the current knowledge, the authors propose a model where CD8+ T cells with the NK-like phenotype could exert pro-inflammatory, regulatory/suppressor or tissue regenerative activities, depending on the cytokine composition of the lung microenvironment. However, they also conclude that further phenotypical and functional studies are necessary for a better classification of these different subtypes of NK-like CD8+ T cells.

De Andrés et al. show original data suggesting that in pregnant women with multiple sclerosis (MS) there is activation of a population CD3+CD8+CD56+ T cells and at the same time a remission of disease activity, akin to an increase in sex hormones. Since this is not observed in non-pregnant women with MS, the data suggest that a subset of regulatory NK-like CD8+ T cells that are activated during pregnancy could play a role in ameliorating MS. However, the factors mediating this activation remain to be elucidated. One of the features of regulatory/suppressor CD8+ T cells is the production of the anti-inflammatory cytokines IL-10 and TGF-β. In this regard, Vuddamalay and van Meerwijk review data comparing classical CD8+CD28− Treg, which express KIR receptors and are therefore NK-like CD8+ T cells, with CD8+CD28low Treg. Although both populations produce IL-10 and TGF-β upon activation, CD8+CD28− Treg originate in the periphery while CD8+CD28low Treg are thought to originate in the thymus. The possible regulatory role of CD8+CD28low Treg in certain immunopathologies is discussed.

Finally, two related articles bring attention to the characterization of innate-like CD8+ T cells in humans and their relevance in cancer. In the first article, and based on their recent identification of a new subset of human NK-like CD8+ T cells expressing KIR, NKG2A, ST2, and Eomes, and rapidly producing IFN-γ upon IL-12/IL-18 triggering, Jacomet et al. provide experimental evidence that chronic myeloid leukemia (CML) is associated with quantitative and functional deficiencies of innate CD8+ T cells that were corrected upon CML remission, suggesting that innate CD8+ T cells may contribute to CML control. In the second paper, Barbarin et al. delineate the putative pathways and signals that could lead to the generation of innate CD8+ T cells capable of controlling cancer development, namely, the cytokines IL-4, IL-12, IL-15, and IL-18, iNKT cells, and the transcriptions factors PLZF and Eomes. In all, these two studies propose that in humans, innate CD8+ T cells constitute a new lymphocyte population that could have an important role in antitumor immunity.

In summary, the articles of this research topic provide novel insights into the mechanisms and conditions that drive the accumulation of NK-like CD8+ in humans and the possible roles played by these multifunctional lymphocytes. Considering their capability of sensing various environmental signals, studies addressing their functional response to physiological challenges, namely, endogenous products released by stressed, injured, or dead cells, will certainly further our understanding into these experienced human CD8+ T cells.

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The author confirms being the sole contributor of this work and approved it for publication.

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REVIEW
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Convergence of Innate and Adaptive Immunity during Human Aging

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Aging is associated with profound changes in the human immune system, a phenomenon referred to as immunosenescence. This complex immune remodeling affects the adaptive immune system and the CD8$^+$ T cell compartment in particular, leading to the accumulation of terminally differentiated T cells, which can rapidly exert their effector functions at the expenses of a limited proliferative potential. In this review, we will discuss evidence suggesting that senescent αβCD8$^+$ T cells acquire the hallmarks of innate-like T cells and use recently acquired NK cell receptors as an alternative mechanism to mediate rapid effector functions. These cells concomitantly lose expression of co-stimulatory receptors and exhibit decreased T cell receptor signaling, suggesting a functional shift away from antigen-specific activation. The convergence of innate and adaptive features in senescent T cells challenges the classic division between innate and adaptive immune systems. Innate-like T cells are particularly important for stress and tumor surveillance, and we propose a new role for these cells in aging, where the acquisition of innate-like functions may represent a beneficial adaptation to an increased burden of malignancy with age, although it may also pose a higher risk of autoimmune disorders.

Keywords: aging, immunosenescence, natural killer receptors, T cell receptor, innate-like T lymphocytes

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INTRODUCTION

Natural killer cells and αβCD8$^+$ T lymphocytes are the two major cell lineages with constitutive cytotoxic activity and have a crucial role in the recognition and killing of abnormal cells. However, the paradigm for the recognition of target cells is fundamentally different between these two cell types: conventional αβCD8$^+$ T cells rely on the T cell receptor (TCR) to recognize specific peptides presented by major histocompatibility complex class-I (MHC-I) molecules, whereas NK cells use a repertoire of germ line-encoded receptors to detect "missing self" or "altered-self" antigens and directly kill abnormal cells, without prior sensitization (1). Besides antigen specificity, the development of immunological memory is conventionally another distinctive feature between NK and T cells, categorizing them into distinct arms of the immune system and the innate and adaptive immune system, respectively (2).

Nevertheless, accumulating evidence supports the existence of NK cell memory (3, 4), as well as evidence for TCR-independent responses mediated by αβCD8$^+$ T lymphocytes (5–7), suggesting that the conventional limits between the innate and adaptive arms of the immune system may be not as distinct as first thought (8). NK and T lymphocytes have a common origin from a lymphoid progenitor cell in the bone marrow (9), and recent comparative proteomic and transcriptomic studies have demonstrated a remarkably close proximity between effector

αβCD8$^+$ T lymphocytes and NK cells (10, 11), reiterating an evolutionary ancestry and shared biology between the two cell lineages.

An increasing body of literature reveals the existence of subsets of T cells with features that bridge innate and adaptive immunity (12–14). In humans, these innate-like T cells comprise the invariant natural killer T (iNKT) cells, CD1d-restricted natural killer T (NKT) cells, mucosa-associated invariant T (MAIT) cells, and γδT cells. These cells typically co-express a TCR and NK cell lineage markers, distinguishing them from NK cells and other innate lymphoid cells (ILCs), which lack the expression of a TCR or somatically rearranged receptors. Functionally, innate-like T cells respond to TCR ligation but are also able to respond rapidly to danger signals and pro-inflammatory cytokines, independently of TCR stimulation, resembling innate cells. Recently, subsets of conventional αβCD8$^+$ T cells expressing NK cell markers and intraepithelial T cells have been included in this vaguely defined group of innate-like T cells (15, 16). Despite the similarities in phenotype and function, there are clear differences in ontogeny and tissue distribution between them.

In this review, we will discuss recent evidence that aging is associated with the expansion of a subset of conventional αβCD8$^+$ T cells with phenotypic, functional, and transcriptomic features that resemble NK cells. Such innate-like αβCD8$^+$ T cells have the characteristics of terminally differentiated T cells, and the acquisition of functional NK receptors is most likely part of a general reprograming of the CD8$^+$ T cell compartment during human aging, to ensure broad and rapid effector functions. We propose that innate-like αβCD8$^+$ T cells share important features with other innate-like T cells; however, fundamental differences in origin and development separate them from truly innate cells. Interestingly, these cells are also differentially affected by aging, suggesting distinct roles in immune responses at different times of life.

IMMUNOSENESCENCE

Aging is associated with a general decline in immune function, contributing to a higher risk of infection, cancer, and autoimmune diseases in the elderly. Such faulty immune responses are the result of a profound remodeling of the immune system that occurs with age, generally termed as immunosenescence (17). While the number of naïve T cells emerging from the thymus progressively decreases with age as a result of thymic involution (18), the memory T cell pool expands and exhibits significant changes in the phenotype and function of antigen-experienced T cells, particularly evident in the CD8$^+$ T cell compartment (19). Chronic immune activation due to persistent viral infections, such as cytomegalovirus (CMV) and Epstein–Barr virus (EBV), is one of the main drivers contributing to the accumulation of highly differentiated antigen-specific CD8$^+$ T lymphocytes that have characteristics of replicative senescence (20, 21). In combination with the depletion of the peripheral pool of naïve T cells, the accumulation of these terminally differentiated T cells with age skews the immune repertoire and has been implicated in the impaired immune responses to new antigens and vaccination in the elderly (22, 23).

The widespread effects of aging on the immune system have been reviewed elsewhere (24) and include defects in the function of natural killer cells, neutrophils, macrophages, and dendritic cells as well as B cells and hematopoietic stem cells. In the innate immune compartment, changes in the phenotype and function of NK cells have been described (25) and associated with the accumulation of CD56^{dim} NK cells with a mature phenotype, characterized by the increased expression of maturation markers, such as CD57 (26) and KLRG1 (27, 28). Although the effects of aging on the cytolytic function of NK cells are still controversial, our group recently identified a subset of CD56^{dim} KLRG1^{high} NK cells that is expanded in the elderly, displaying impaired cytotoxicity and proliferation as well as other features of senescence (28).

While many aspects of the immune response are impaired, there is also evidence for hyperresponsiveness of the immune system during aging (29). It is likely that there is a complex remodeling of the immune system throughout life in an attempt to maintain effective immune responses, which could be beneficial in the responses to infections and cancer but may carry an increased risk of autoimmune and inflammatory diseases in the elderly (30).

CHARACTERISTICS OF HIGHLY DIFFERENTIATED αβCD8$^+$ T CELLS

Multiple phenotypic and functional features have been proposed to define senescent CD8$^+$ T cells (**Table 1**). Loss of co-stimulatory receptors, such as CD28 and CD27, is one of the most consistent immunological markers of T cell aging (31, 32) which, in combination with other markers of maturation such as CD45RA, KLRG1, and CD57 expression, identifies a subpopulation of long-lived immune cells with characteristics of terminal differentiation or senescence (33).

Several lines of evidence indicate that end-stage CD27$^-$ CD28$^-$CD45RA$^+$ CD57$^+$ T cells accumulate significantly in older humans (34), during chronic viral infections (35) and in chronic inflammatory diseases (36). These cells exhibit the characteristic features of senescence that include accumulation of DNA damage markers, short telomeres, low proliferation, and loss in the capacity to activate the enzyme telomerase (37–39). A paradoxical observation is that senescent CD8$^+$ T cells maintain potent effector functions, despite the loss of proliferative capacity, and thus should not be considered as a residual population of dysfunctional cells. On the contrary, these cells are polyfunctional, reflecting their ability to simultaneously carry out multiple functions, including secretion of IFN-γ and TNF-α and cytotoxicity (35, 38, 40), and this is an important observation that distinguishes senescent from exhausted T cells (41). Nevertheless, the increased secretion of pro-inflammatory cytokines by senescent T cells may have detrimental effects on the tissue microenvironment and contribute to the age-associated low-grade inflammatory state termed "inflammaging" (42).

Highly differentiated T cells have impaired TCR signaling (43, 44). We recently described that senescent CD27-28$^-$ CD4$^+$ T cells exhibit decreased expression of key components of the TCR signalosome, such as LCK, LAT, and SLP-76 (39), and found

TABLE 1 | Phenotypic and functional characteristics of senescent CD8$^+$ T cells, compared to less differentiated subsets.

	Early differentiation	Intermediate differentiation	Terminal differentiation
Phenotypic markers			
CD28	++	+/−	−
CD27	++	+/−	−
CD45RA	++	+/−	+/−
CCR7	++	+	−
CD62L	++	+	−
CD57	−	+/−	++
KLRG1	−	+/−	++
Other NKR (KIR, NKG2, and CD56)	−	+/−	++
Functional features			
Proliferation	++	+	−
Telomerase activity	++	+	−
Telomeres	+++	++	+
Cytotoxicity	−	+	++
Cytokine secretion (TNF-α, IFN-γ)	−	+	++
Signaling pathways			
TCR signaling	+	++	+/−
IL-2 signaling	+	++	+/−
Pi3K–AKT–mTOR signaling	+	++	+/−
p38MAPK activation	−	−	+

KLRG1, killer cell lectin-like receptor G1; NKR, natural killer receptor; KIR, killer cell immunoglobulin-like receptor; NKG2, natural killer receptor G2, TNF-α, tumor necrosis factor alpha; IFN-γ, interferon gamma; PI3K, phosphatidylinositol-3 kinase; mTOR, mammalian target of rapamycin.

similar observations in end-stage CD8$^+$ T cells. Interestingly, as T cells progressively differentiate, they concomitantly start expressing NK lineage receptors. Collectively, these observations suggest that, as CD8$^+$ T cells terminally differentiate, they become less dependent on antigen-specific signals and more responsive to innate-like signals.

TCR HYPORESPONSIVENESS IN TERMINALLY DIFFERENTIATED T CELLS

Conventionally, optimal activation of T cells requires the engagement of the TCR and the second signal usually delivered by co-stimulatory receptors or cytokines. However, as previously mentioned, T cell senescence is associated not only with the loss of co-stimulatory receptors but also with impairment of TCR signaling (43, 44), leading to defects in classical T cell functions.

Changes in the composition of membrane lipids and lipid rafts have been described and linked to the age-related changes in TCR proximal signaling (45). More recently, Li and colleagues investigated the molecular mechanisms accounting for the loss of TCR sensitivity with age and found a correlation with the decreased expression of miR-181a and increased activity of DUSP6, a phosphatase that negatively regulates proximal TCR signaling (46). We recently demonstrated that the accumulation of DNA damage in senescent CD4$^+$ T cells leads to the activation of AMP kinase, which is implicated in the decreased expression of key elements of the proximal TCR machinery, leading to impaired proximal TCR signaling in these cells (39). It is evident from these studies that aging is associated with a decrease in TCR responsiveness. Interestingly, recent studies have linked the acquisition of innate-like effector functions by memory CD8 T cells with defective TCR signaling (47–49).

TCR-INDEPENDENT ACTIVATION OF αβCD8$^+$ T CELLS

Accumulating evidence indicates that memory CD8$^+$ T cells may be activated in a TCR-independent manner through a process called bystander activation. This occurs in the absence of the cognate antigen, through the action of inflammatory cytokines, such as type I interferons (50, 51), IL-15 (52), IL-12 (53), IL-18, or a combination of these (5, 7, 54).

In addition to inflammatory cytokines, the acquisition of stimulatory innate immune receptors has been implicated in antigen-independent activation of CD8$^+$ T cells. Among them, C-type lectin activating receptors, such as NKG2D and NKG2C, which recognize self-ligands related to the MHC-I have been shown to play crucial role in the mediation of innate-like responses by CD8$^+$ T cells (6, 55). NKG2D is a classical example of a NK cell receptor that is highly expressed on αβCD8$^+$ T cells and subsets of γδ T cells (56, 57). While the general consensus is that NKG2D engagement serves as a co-stimulatory receptor in CD8$^+$ T cells, amplifying TCR signals in virus-specific responses (58) as well as antitumor immunity (59, 60), other studies have provided compelling evidence that CD8$^+$ T cells may respond to NKG2D ligation alone, without TCR engagement, provided that cells are stimulated with cytokines, such as IL-15 or high doses of IL-2 (6, 49, 61, 62). Such TCR-independent, NKG2D-dependent mechanism of activation of CD8$^+$ T cells has been shown important for host defense against infections (49) and tumor surveillance (63) but has also been implicated in the pathogenesis of inflammatory and autoimmune reactions (6).

Collectively, these findings challenge the classic paradigm that TCR engagement by the cognate antigen is necessary for the activation of T cells and support the role of innate-like receptors in the regulation of T cell effector functions. Overall, such observations may shed light on the question of how senescent T cells maintain potent effector functions, despite the TCR hyporesponsiveness.

EXPANSION OF αβCD8$^+$ T CELLS EXPRESSING NK CELL RECEPTORS WITH AGING

Studies in human centenarians have shown an increased proportion of T cells expressing NK cell receptors (NKRs), whereas these cells represent a minor population of circulating lymphocytes in newborns and young healthy individuals (64, 65). The frequency of NKR-expressing T cells not only increases with age but also in conditions associated with chronic immune activation (66–68). Among the most commonly observed NKR on T cells are activating and inhibitory receptors, such as CD16, CD56,

CD57, NKp30, KLRG1, and CD94, members of the NK receptor G2 (NKG2), and killer-cell immunoglobulin-like receptor (KIR) families (10, 66–69).

Phenotypic analysis of NKR-expressing T cells revealed that the majority of these cells are highly differentiated effector memory CD8$^+$ T cells, lacking CD28 expression and exhibiting other features of senescence (62, 69–71). Importantly, it has been demonstrated that these cells derive from conventional αβCD8$^+$ T cells (71), express an oligoclonal αβTCR, and do not express the semi-invariant TCR Vα24/Vβ11 chains, excluding that they represent an expansion of the classical iNKT cells (17).

A recent study using single-cell mass cytometry to analyze the expression of NKR across the human immune system found, as expected, an increased expression of NK cell markers on CD8$^+$ T cells, more evident in individuals with high levels of CD57, indicative of a terminally differentiated immune system (10). As the immune system matures, the diversity of the NKR repertoire increases on both NK and CD8$^+$ T cells; however, the difference in magnitude for the gain of activating receptors appears to be much higher in CD8$^+$ T than in NK cells. Hierarchical clustering based on NKR expression patterns unexpectedly clustered CD8$^+$ T cells closer to mature NK cells than to CD4$^+$ T cells.

Although the expansion of NKR-expressing T cells is mostly evident in the CD8$^+$ T cell compartment, the expression of NK cell markers has also been found on human CD4$^+$ T cells. For instance, our group and others have identified a subset of highly differentiated CD4$^+$ T cells expressing NKG2D as well as cytotoxic granules, expanded in aging (35, 72) and autoimmune diseases (73, 74).

What triggers the expression of NKRs on T cells with aging is not yet clearly defined. TCR engagement and cytokine stimulation have been shown to induce the expression of NKRs on T cells both *in vitro* and *in vivo* (75, 76). In addition, studies in transplant recipients have demonstrated a striking upregulation of NKR in virus-specific CD8$^+$ T cells after CMV reactivation (77), suggesting that chronic antigenic stimulation may drive the expansion of NKR-expressing T cells. Likewise, the upregulation of inhibitory NKRs, such as NKG2A and KLRG1, has been linked to clonal expansion after antigenic exposure and development of replicative senescence of T cells (20, 34, 78, 79).

REPROGRAMING OF SENESCENT αβCD8$^+$ T CELLS INTO INNATE-LIKE T CELLS

The biological significance of NKR acquisition on CD8$^+$ T cells during aging is not yet fully understood. It remains unclear whether the expansion of NKR-expressing T cells with age is a stochastic effect associated with chronic antigenic stimulation or whether it represents a predetermined program to allow these cells to respond rapidly in an innate-like fashion.

Functional studies performed with human CD8$^+$ T cells that were activated and expanded *ex vivo* in the presence of cytokines or after TCR cross-linking, revealed that the acquisition of an NK cell phenotype was generally associated with the acquisition of functional features characteristic of NK cells (75). Of particular note, these cytokine-induced killer (CIK) cells develop the capacity to mediate MHC-unrestricted killing of target cells, in particular tumor cells, identifying them as potential tools in cancer therapy (80). Such activity does not require prior antigenic exposure but involves the engagement of stimulatory NKR and prior stimulation with inflammatory cytokines. Interestingly, these cells display a duality of function, as they are able to mediate both TCR-independent and antigen-specific immune responses (81).

Gene-expression studies have greatly contributed to dissecting the transcriptional changes occurring in aged T cells and shed light on the significance of NKR acquisition [reviewed in Ref. (82)]. Fann and colleagues originally compared the gene-expression profiles of human CD28^{null} and CD28$^+$ memory CD8$^+$ T cells and found significant changes in the CD28^{null} compartment, such as (1) decreased expression of co-stimulatory receptors, (2) acquired expression of NKRs (the majority of which have stimulatory activity), (3) upregulation of genes involved in cytotoxicity (in particular genes involved in the granule exocytosis pathway, perforin and granzymes, and in the Fas ligand/Fas pathway), (4) elevated expression of chemokines and cytokine receptors, and (5) differentially expressed signaling molecules and transcription factors (83). Subsequent studies comparing gene-expression profiles of CD8$^+$ T cells between young and old donors have found similar changes, particularly in relation to enhanced expression of genes in the NK cell cluster (84, 85). Of particular note, Cao et al. described additional changes at the level of cell signaling pathways in aged CD8$^+$ T cells, the most prominent involving an age-decreased expression of genes associated with TCR, IGF-1, and PI3K/AKT signaling pathways (85). Collectively, these studies point to a common transcriptional signature in aged CD8$^+$ T cells that most likely reflect the acquisition of potent cytotoxic effector functions, largely independent of TCR signals.

It remains to be determined which transcriptional factors are the main regulators of this program. The differential expression of T-box transcription factors, T-bet and eomesodermin (Eomes) in aged T cells compared to the less differentiated counterparts (83), suggests a role in the reprograming of senescent CD8$^+$ T cells. However, several other transcriptional regulators have been implicated in the terminal differentiation of cytotoxic CD8$^+$ T cells, including the Foxo family of transcription factors [reviewed in Ref. (86), Blimp-1 (87, 88), ZEB2 (89), and promyelocytic leukemia zinc finger (PLZF) (90)]. Interestingly, some of these factors have been also implicated in the transcriptional control of NK and NKT cell differentiation (91, 92), and PLZF has been proposed as the key determinant factor for the development of innate T cells (92, 93). More importantly, overexpression of PLZF in conventional T cells was sufficient for the acquisition of innate-like phenotype and functions (90). Many questions remain in regard to the transcriptional program underlying T cell senescence. Importantly, it remains to be determined which factors control the peripheral modulation of TCR signaling and whether there is a mechanistic link between the acquisition of NKRs and the modulation of the TCR machinery.

Collectively, these observations indicate that the acquisition of receptors that are normally found on NK cells may be part of a general reprograming of CD8⁺ T cells with maturation (**Figure 1**). Not only these cells acquire phenotypic markers of NK cells but also they acquire innate-like cytolytic functions, suggesting that a coordinated transcriptional program endows these cells with the machinery to respond to innate stimuli, without the requirement for TCR activation.

SIMILARITIES AND DIFFERENCES WITH INNATE-LIKE T CELLS

Innate-like T cells are phenotypically characterized by the co-expression of a TCR with conventional NK cell lineage markers. NKT cells are the prototypical example of innate T lymphocytes. The term NKT cell is sometimes misused to refer to other subsets of conventional αβT cells that express NKRs, although there are fundamental differences between them. Classical NKT cells express an invariant TCR (Va24Ja18) that recognize glycolipids presented by the monomorphic CD1d molecule, and they account for 0.1–1% of T cells in human peripheral blood (94), whereas conventional αβT cells expressing NKR exhibit a diverse TCR repertoire and their frequency in peripheral blood is much higher, increasing with age and chronic inflammatory diseases (68). In striking contrast, aging is associated with decreased frequency and function of iNKT cells (95, 96).

Recently, it has been proposed that the suppression of TCR signaling is critical for the development of innate-like T cells. An elegant study done by Hayday and colleagues in mice models has brought some insights into how innate T cells downmodulate the TCR signaling machinery to allow an innate mode of activation in peripheral tissues, independent of the TCR (97). The authors demonstrated that this mechanism of TCR tuning after development in the thymus, concomitant with acquisition of responsiveness to innate signals is a feature shared by diverse subsets of innate-like T cells, including CD27⁻ γδT cells, mouse dendritic epidermal T cells (DETCs), and intestinal epidermal TCRαβ⁺ and γδ⁺ T cells. Although they could not find a similar mechanism to occur in iNKT cells, another study has

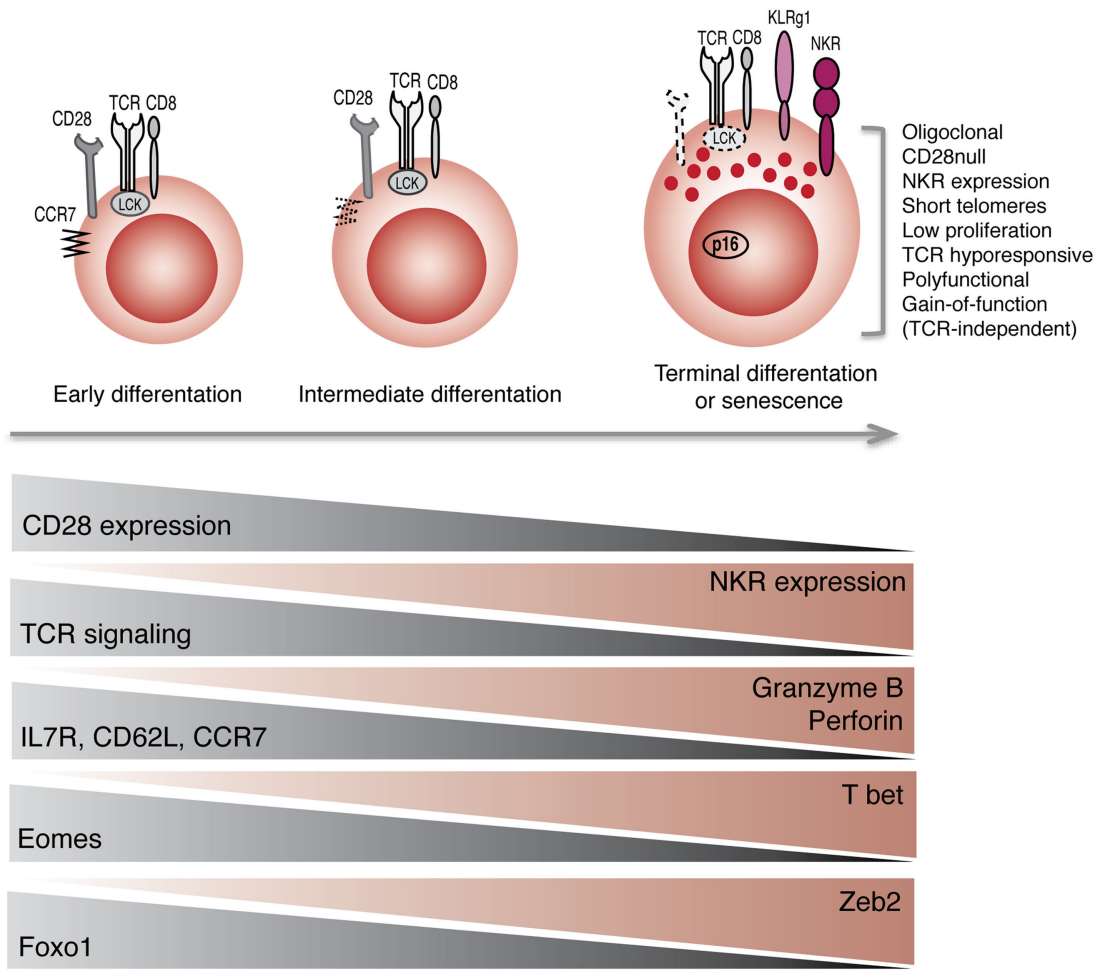

FIGURE 1 | **Reprograming of CD8⁺ T cells into NK-like T cells with aging**. Progression toward terminal differentiation or senescence is associated with phenotypic, functional, and transcriptional changes that lead to the expansion of NK-like CD8⁺ T cells.

demonstrated the acquisition of transient innate responsiveness by human iNKT cells *via* histone modifications induced by weak TCR stimulation (98).

Functionally, it has been demonstrated that innate-like lymphocytes are able to respond to TCR ligation as well as to innate signals alone, in particular to NKG2D ligation and to inflammatory cytokines (99–101). In humans, conventional αβCD8$^+$ cells in celiac disease have been shown to respond to NKG2D ligands and pathological levels of IL-15, independently of TCR ligation (6). It remains to be determined if TCR signaling is suppressed in these cells, suggesting a common signature with other innate-like T cells.

Collectively, the observations that terminally differentiated CD8$^+$ T cells co-express NKR and TCR have decreased TCR responsiveness and yet are able to respond rapidly to stimulation, without the requirement for cognate antigen supports the hypothesis that human senescent αβCD8$^+$ T cells exhibit phenotypic and functional features that resemble other innate-like T cells. Nevertheless, the origin and development of human senescent αβCD8$^+$ T cells is distinct from that of classical innate T cells. While innate T cells are developmentally pre-programed in the thymus (12), αβCD8$^+$ T cells with innate-like features arise in the periphery, most likely as a result of a general reprograming driven by external environmental cues. The different origin may explain why aging is associated with a decreased frequency of innate T cells such as NKT cells, as a result of thymic involution, whereas the number of conventional αβT cells expressing NKR increases in the elderly, most likely a result of the homeostatic redistribution of T cells to compensate for the decrease in the output of T cells from the thymus with age.

PHYSIOLOGICAL ROLE OF INNATE-LIKE αβCD8$^+$ T CELLS

The capacity to mediate dual innate and adaptive immune functions place senescent αβCD8$^+$ T cells alongside other innate-like cells in the frontline of defense against pathogens and tumors. The acquisition of innate sensors specialized in the recognition of "danger" signals allows these cells to switch to a rapid and efficient mode of action in potentially harmful situations. Given the increased burden of tumors and infections with age, the contribution of such innate-like CD8$^+$ T cells may be crucial. The capacity to mediate MHC-unrestricted killing against a broad array of tumor targets has been demonstrated *in vitro* and *in vivo* with CIK cells putting these cells as attractive candidates for immunotherapy in solid organ and hematopoietic cancer treatment (102). Interestingly, despite showing a decreased TCR responsiveness, studies indicate that these cells still retain the capacity to elicit specific TCR-dependent immune responses (81).

Nevertheless, the reversal of antigen-specific CD8$^+$ T cells to an innate mode of function is not without consequence. The peripheral requirement for TCR engagement for T cell activation is an important control mechanism to prevent autoreactivity. In conditions associated with chronic activation and inflammation, the balance between activating and inhibitory signals may favor the onset of autoimmune reactions. Recent reports have demonstrated a role of NKG2D in CD8$^+$ T cell activation in inflammatory states and other stress conditions where NKG2D ligands are induced in normal tissues, such as celiac disease (6), type I diabetes (103), and transplantation (104, 105).

CONCLUDING REMARKS

In this review, we summarize evidence indicating that chronological aging is associated with accumulation of cells combining features of both the innate and adaptive arms of the immune system, most likely to compensate for functional defects of conventional NK and CD8$^+$ T cells with age. We propose that senescent CD8$^+$ T cells should not be seen as a dysfunctional population but instead a functionally distinct subset, which uses recently acquired NK cell machinery to maintain rapid effector functions throughout life. Contrary to the classic paradigm that peripheral TCR ligation is essential for T cell activation, this subset of highly differentiated T cells has impaired TCR responsiveness and may be non-specifically activated by inflammatory cytokines or after ligation of innate receptors. The switch to an innate mode of function may shed light on the mechanisms that allow highly differentiated CD8$^+$ T cells to maintain they polyfunctionality, despite the loss of TCR signalosome.

Our understanding of the physiological significance of the expression of NKRs on T cells is still incomplete, and the identification of the molecular mechanisms and the transcriptional regulators underpinning the development of innate features in T cells is essential. Most importantly, it will be important to understand how the intersection between innate and adaptive immune features may be manipulated to enhance immune function and to use this information to develop new approaches to improve immunity in the elderly.

AUTHOR CONTRIBUTIONS

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Functionally Diverse NK-Like T Cells Are Effectors and Predictors of Successful Aging

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The fundamental challenge of aging and long-term survivorship is maintenance of functional independence and compression of morbidity despite a life history of disease. Inasmuch as immunity is a determinant of individual health and fitness, unraveling novel mechanisms of immune homeostasis in late life is of paramount interest. Comparative studies of young and old persons have documented age-related atrophy of the thymus, the contraction of diversity of the T cell receptor (TCR) repertoire, and the intrinsic inefficiency of classical TCR signaling in aged T cells. However, the elderly have highly heterogeneous health phenotypes. Studies of defined populations of persons aged 75 and older have led to the recognition of successful aging, a distinct physiologic construct characterized by high physical and cognitive functioning without measurable disability. Significantly, successful agers have a unique T cell repertoire; namely, the dominance of highly oligoclonal αβT cells expressing a diverse array of receptors normally expressed by NK cells. Despite their properties of cell senescence, these unusual NK-like T cells are functionally active effectors that do not require engagement of their clonotypic TCR. Thus, NK-like T cells represent a beneficial remodeling of the immune repertoire with advancing age, consistent with the concept of immune plasticity. Significantly, certain subsets are predictors of physical/cognitive performance among older adults. Further understanding of the roles of these NK-like T cells to host defense, and how they integrate with other physiologic domains of function are new frontiers for investigation in Aging Biology. Such pursuits will require a research paradigm shift from the usual young-versus-old comparison to the analysis of defined elderly populations. These endeavors may also pave way to age-appropriate, group-targeted immune interventions for the growing elderly population.

Keywords: CD16, CD56, cell senescence, functional performance, immune remodeling, NKG2D, plasticity, TCR-independent

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Abbreviations: 3MS, average of three tests of the modified minimental examination; ADL, activities of daily living; GMFI, geometric mean fluorescence intensity; IFN, interferon; IL, interleukin; TCR, T cell receptor; TNF, tumor necrosis factor.

INTRODUCTION: ALTERATIONS IN CLASSICAL T CELL-MEDIATED IMMUNITY DURING AGING

Studies comparing young and old humans and mice have led to a voluminous body of literature showing a general age-related decline in various physiologic functions. In the immune system, among the most notable age-dependent physiologic retrogressions in the T cell compartment are inefficiencies in classical T cell receptor (TCR) signaling, thymic involution, contraction of the naïve T compartment, expansion of the memory T cell compartment, and overall shortening of telomeres (1–8). At the cellular level, aged $CD4^+$ and $CD8^+$ T cells have a deficiency in the expression of CD28 that coincides with highly shortened telomeres, high levels of expression of mitotic inhibitors, such as p16 and p53, and a severe limitation or complete lack of mitotic activity (9–13). All of these alterations have been argued to underlie the relative poorer antigen-specific T cell-dependent immunity among older adults compared to younger persons.

HETEROGENEITY OF PHENOTYPES OF OLDER ADULTS

Older adults (generally defined as those aged ≥65 years), however, have highly heterogeneous health and immune phenotypes. They range from the frail and chronically ill residents of long-term care facilities to the community dwellers that are living independently (14–17). Many of them retain their ability to mount vaccine responses, including to the pandemic and seasonal influenza vaccines, and to the zoster vaccine (18–22). There are evidences of functionally active virus-specific T cells during new and reactivated latent infections (23–25). Old age has also become less of hurdle in the setting of organ transplantation for either organ donors or recipients (26–29). Thus, aging is not synonymous with poor health, or that the elderly are not mere defective versions of the young.

Heterogeneity of older adults provides a compelling rationale for a re-appraisal of "immunosenescence." In its current usage, the term refers to the poorer degree of immune responsiveness of older adults relative to that seen in the young, a generalized and vague definition that has not substantially differed from the original concept proposed by Walford in the 1950s (30). Learning from epidemiological and geriatric studies (14–17, 31), we have articulated the paramount importance for the analysis of defined populations of the elderly, instead of continuing with the usual young-versus-old comparative approach. Such research paradigm shift is a key toward unraveling immunopathways that underlie discrete physiologic constructs of aging, such as frailty and successful aging (32, 33).

IRREVERSIBLE LOSS OF CD28: A SIGNATURE OF AGING IN HUMAN T CELLS

CD28 is the major co-stimulatory molecule that is required to sustain normal T cell activation (34) and for the elaboration of antigen-specific effector function in both naïve and memory compartments (35–37). In cohort studies, we provided the definitive proof for progressive loss of CD28 with chronologic aging (12). Such loss or absence of CD28 has long been thought to lead to deficiency or inefficiency of TCR signaling in aged T cells (10, 38). Indeed, mice with homozygous deletion of *CD28* results in an immunosuppressed phenotype, since mouse $CD28^{-/-}$ T cells are anergic and prone to activation-induced cell death (35, 39).

The loss of CD28 on human T cells with aging (10, 12, 40) may not be surprising since CD28 expression is subject to transient downregulation during a normal immune response (41). In fact, deficiency of its expression is characteristic of continuous passages of T cell cultures (40, 42). These unusual $CD28^{null}$ $CD8^+$ T cells have shortened telomeres (13), consistent with telomere-dependent senescence (sometimes referred to as "replicative senescence") akin to those reported for other human somatic cells (43–47).

Due to more rapid turnover, $CD8^+$ T cells have higher rate of CD28 loss than $CD4^+$ T cells (48, 49). $CD28^{null}$ $CD4^+$ and $CD8^+$ T cells are highly oligoclonal and have highly shortened telomeres, indicating their long replicative history (12, 13). They also have high expression levels of p16 and 53, and they have limited, if not complete lack of, proliferative capacity even under conditions of optimal stimulation *via* TCR/CD3 in the presence of interleukin (IL)-2 *in vitro* (11, 12, 50, 51). All these properties are consistent with replicative senescence.

CD28 loss and telomere shortening are properties of primates, being typical of elderly humans as described above, as well as for older macaques and other anthropoids (52–55). In contrast, mouse T cells maintain long telomeres, and neither $CD4^+$ nor $CD8^+$ T cells show perceptible telomere shortening with multiple cell divisions *in vitro* (56). Indeed, it takes at least four generations for the telomerase-deficient mouse to show quantitative shortening of telomeres (57), indicating mice clearly do not undergo telomere-dependent replicative senescence.

Clonal expansions of T cells are characteristic of old mice similar to old humans (58). However, mouse T cells do not lose CD28 expression with chronologic aging. In fact, CD28 expression level may actually increase with age (59). Such species-specific difference in CD28 expression pattern between humans and mice is attributable to entirely non-homologous DNA sequences in the promoter regions of the *CD28* gene (60) (*Homo sapiens* CD28, NCBI Gene 940, HGNC 1653; *Mus musculus* CD28, MGI 88327, NCBI Gene 12487). These age-related loss/maintenance of telomeres and loss of CD28 underscore that transposition of data obtained from mouse studies to human biology is unsound. We have articulated that while aging mouse models are instructive about the general biology of aging, they do not substitute for analytical studies of human elderly subjects (61).

The loss of CD28 is generally irreversible, due to the direct inactivation of the gene promoter (42, 62). The transcriptional initiator, a DNA sequence module in the 5′ *cis*-acting *CD28* regulatory region where the activator complex, including nucleolin and heterogeneous ribonucleoprotein-DOA, is unoccupied in senescent $CD28^{null}$ T cells (63). Nucleolin and heterogeneous ribonucleoprotein-DOA are found in senescent T cells, but they do not form a functional initiator complex. While mechanism(s)

underlying the failure of the assembly of this transcriptional complex remains to be investigated, it is clear that non-occupancy of the *CD28* initiator results in a transcriptional block, leading to the absence of all splice forms of *CD28* mRNA and the lack of expression of CD28 on the T cell surface (42, 64, 65).

CD28^{null} T cells are resistant to apoptosis (66), which explains their persistence in circulation for years and their pervasive accumulation *in vivo* with advancing age. This is attributed to constitutively high levels of expression of Bcl2 and Bcl-xL, with corresponding downregulation of Bax (12, 67). Bcl-independent pathways for the lifelong persistence of these cells have also been reported (68).

DE NOVO EXPRESSION OF NK-RELATED RECEPTORS ON CD28^{null} T CELLS: FUNCTIONAL DIVERSITY AND VERSATILITY OF AGED T CELLS

Whether they are naturally derived *in vivo* during aging, or in an *in vitro* senescence system, oligoclonal senescent CD28^{null} T cells have a unique phenotype for their *de novo* acquisition of a diverse array of receptors normally expressed on NK cells (12, 50, 69, 70). The repertoire of NK-related receptors they express does not reflect the full complement of the many *NK receptor* genes normally expressed on NK cells (50). However, the NK-related receptors on aged CD28^{null} T cells are expressed co-dominantly in varying combinations along clonal lineages. CD28^{null} T cells with identical *TCR CDR3* sequences, indicating their common origin from a single mother CD28^{+} T cell, may express different types of NK-related receptors (71, 72).

Whether the loss of CD28 is required for, or is an event independent from, the expression of NK-related receptors remains to be examined. However, it is clear that differences in the patterns of expression of these receptors between NK cells and CD28^{null} T cells are related to cell-specific differences in the regulatory modules of each NK-related receptor. For example, we have shown that differential expression of CD158b1 (KIR 2DL2) between T and NK cells are controlled by two distinct transcriptional regulatory motifs on the upstream *cis*-acting promoter region of the gene; namely, a proximal element at −51 and an AML site at position −98 for T and NK cells, respectively (73). Other investigators have reported the role of age-related epigenetic alterations. Differential induction of CD158d (KIR 2DL4) and CD158b2 (KIR 2DL3) on T cells is related to methylation/demethylation on promoter regions of these two genes, in contrast to their classical promoter-driven expression as seen in NK cells (74–76). These studies suggest that there may be diverse regulatory machineries involved in the induction of NK-related receptors on T cells with aging. Given the diversity of these receptors and their apparent co-dominant expression, it will be of interest to examine whether and how expression of one NK-receptor affects the expression of another NK-receptor during the aging process. A particular interest is the regulation of expression of the prototypic receptors CD56, CD16, and NKG2D on aged T cells. But regardless of whether such regulation occurs at the level of unique promoter motifs, or through structural alterations of chromatin that favor accessibility of the particular *NK-receptor* gene, or perhaps through posttranscription controls, it is clear that the acquisition of NK receptors by T cells corresponds with the elaboration of new effector function (77).

The phenomenal age-related expression of NK-related receptors on T cells has been associated with seropositivity to cytomegalovirus (CMV) (78). This is in line with reports about similar association of CMV serology with frequency of CD28^{null} T cells, and such serological-cellular association has been argued to be a predictor of poor health outcomes of aging (79, 80). It has also been suggested that CMV infection may lead to the emergence of these senescent T cells that are considered dysfunctional or non-functional (81–83). However, such studies are purely associational rather than causal. Further, the association is not universal. The cited studies are mostly from those on elderly populations in Northern Europe where CMV exposure appears to occur gradually over the life span, which might explain the high CMV seropositivity in old age (80). In the United States, CMV exposure is already widespread at early adolescence (84). Yet, we have shown that senescent NK-like T cells are rarely found among young Americans (12, 32). Importantly, we found very high titers of anti-CMV antibody among older adults and found no clinical evidence of CMV disease. Indeed, another cohort study showed CMV seropositivity alone is an insufficient measure of health risk among older Americans (85). In addition, populations of CMV-specific T cells have been found to be functional with clear beneficial antiviral effects (68, 86, 87). A recent experimental study has shown further that CMV by itself does not induce replicative senescence for T cells (25). CMV disease is undoubtedly serious whether it happens at an early or old age. However, the causative role of CMV in human T cell senescence is yet to be proven. Broader experimental studies are needed to determine what particular environmental and/or endogenous factors trigger, drive, and maintain populations of senescent NK-like T cells *in vivo* during the aging process.

The array of NK-related receptors expressed on aged CD28^{null} T cells is summarized in **Figure 1**. They include the prototypic stimulatory NK receptors, CD16, CD56, and NKG2D. They may also express CD161, and various inhibitory NK receptors such as CD94 and NKG2A, and members of the CD158 killer cell immunoglobulin-like receptor family (12, 50, 69, 72, 77, 88–90). Unlike the selective single allelic expression for TCR, NK-related receptors are expressed co-dominantly on aged T cells.

In addition to shortened telomeres, high p16/53 expression levels, and irreversible loss of CD28, aged NK-like T cells express two other markers of senescence, namely, KLRG1 and CD57 (81, 91, 92). KLRG1 is an inhibitory NK-related receptor that has been shown to actively suppress classical TCR signaling (93). CD57 is an adhesion molecule that is typically expressed on terminally differentiated T cells. Although it is still unclear if CD57 itself is a signaling receptor that dictates or alters T cell effector function, its expression on T cells is biomarker for cell cycle arrest in aged T cells (91, 94). It might be noted that CD57 is also expressed on highly differentiated NK cells (95, 96). However, whether such CD57^{+} NK cells are senescent, and that CD57 directly controls NK cell function are also not yet known.

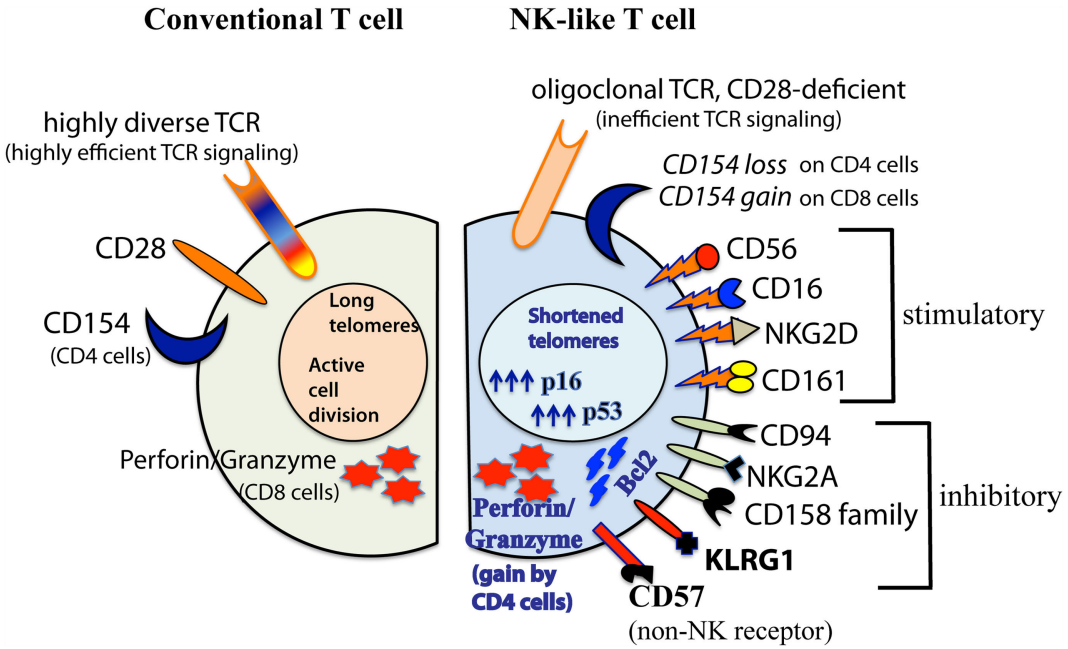

FIGURE 1 | Diagrammatic comparison between conventional CD28^{+} and senescent CD28^{null} NK-like T cells. This illustration summarizes findings from various investigators as described in the text. The diagram shown was modified from (32) with permission from *Aging and Disease* journal under the terms of Creative Commons Attribution License (CC BY). This license allows the unrestricted use, distribution, modification, and reproduction in any medium by the author with credited citation of the original publication.

Despite their senescent properties, CD28^{null} NK-like T cells are highly functional and versatile. While there is general trend for the varying inefficiencies of classical TCR signaling during aging (1, 2, 97–99), there could still be residual TCR signaling as exemplified by long-lived memory T cells in the context small pox and polio vaccination (100, 101). Indeed, experimental studies showing unusually high constitutive level of expression of interferon (IFN) γ in CD28^{null} T cells can further increase following ligation of TCR/CD3 (38, 94, 102). Such residual TCR-driven response may be attributed to other co-stimulatory molecules, such as 41BB ligand, OX40, CD70, and CD58, which substitutes for the defunct CD28 (103–107).

More significantly, we have reported that effector activities of CD28^{null} NK-like CD4^{+} and CD8^{+} T cells are directly attributable to signaling of the NK-related receptors they express in a totally TCR-independent manner (12, 70). We have shown that CD56-driven and NKG2D-driven expression of the early activation cell surface antigen CD69; the intracellular expression of IL-4, IFNγ, CD107b/LAMP2, perforin, and granzyme; and the late cell surface expression of exocytosis protein CD107a are to be as effective as, if not better than, classical TCR stimulation. In fact, the CD56-/NKG2D-driven TCR-independent expression of perforin, granzyme, and CD107a occur in both CD4^{+} and CD8^{+} NK-like T cells. This indicates that the conventional "helper" and "cytotoxic" designations for CD4^{+} and CD8^{+} T cells, respectively, are not instructive about of the biology of T cells in old age. Similarly, the expression of CD154 (CD40 ligand) on aged T cells does not follow the usual CD4 helper paradigm. CD154 is lost on senescent CD28^{null} CD4^{+} NK-like T cells but is gained by senescent CD28^{null} CD8^{+} NK-like T cells (38, 108, 109). This suggests that the latter cell subset is a potential target to boost humoral immunity in the elderly.

AGE-DEPENDENT ACCUMULATION OF CD28^{null} NK-LIKE T CELLS WITH OLIGOCLONAL TCRs: IMMUNE REPERTOIRE REMODELING CONSISTENT WITH PHYSIOLOGIC PLASTICITY IN OLD AGE

Physiologic systems are optimized toward reproduction, after which the goal is individual survival (110). There is evolutionary conservation of biological pathways that ensure individual survival beyond reproductive maturity (111, 112), including a variety of genes referred to as "longevity assurance" genes that promote long-term survival (113–116). Older organisms are essential in maintaining population structures particularly among social animals and are therefore involved ultimately in the perpetuation of the species (112, 117–119).

Immunity is an evolutionary determinant of individual fitness and survival (120–122). The accumulation of NK-like CD28^{null} T cells with advancing age represents a remodeling of the immune repertoire as a compensatory mechanism for the general age-related losses in conventional T cell-dependent immunity (123). As described previously, there is thymic atrophy with age leading to impaired production of new naïve T cells, making older adults unable to respond to new and emerging pathogens

in an antigen-specific manner (3, 124). With antigenic exposure through life, there is progressive contraction of the naïve T cell compartment, with corresponding expansion of memory and senescent T cell compartment. These events over the lifespan result in the contraction of diversity of the clonotypic TCR repertoire (5, 49). With cycles of expansion and death of T cells during antigenic challenges, the phenomenal accumulation of apoptosis-resistant CD28^{null} NK-like T cells is likely a protection against clinical lymphopenia, which is very rare among older adults (125, 126).

The acquisition of a diverse array of NK-related receptors on CD28^{null} T cells maintains immunologic diversity in old age. As discussed previously, there is co-dominant expression of diverse NK-related receptors along clonal lineages of CD28^{null} T cells in late life. This is in stark contrast to the conventional clonotypic TCR diversity that is characteristic of the young. Signaling of these NK-related receptors effectively imparts an innate function to aged T cells (12, 70); hence, we had originally introduced the term "NK-like T cells" to emphasize their NK-related receptor-driven, TCR-independent effector function (50). The term underlines the diverse array of NK-related receptors expressed along oligoclonal TCRαβ lineages, in contrast to convention αβTCR repertoire diversity in the young (12). NK-like T cells are distinct from conventional NKT cells (or invariant iNKT cells), which are identified a single invariant TCR AV24BV11 that recognizes glycolipid antigens presented in the context of CD1d instead of conventional HLA antigen-presenting molecules (127, 128).

NK-like T cells compensate for the corresponding age-related functional loses in the NK cell compartment (32). NK cell numbers are largely maintained through life, but skewing of certain NK cell subsets with aging have been reported (129). We have shown that octo-/nona-genarians have contracted pools of CD56^{+} and CD16^{+} NK cells, which are accompanied by corresponding age-dependent gains of CD56 and CD16 expression on both CD4^{+} and CD8^{+} T cells (32, 70). As already described previously, CD56 ligation alone can drive T cell effector activities. The function of CD16 on NK-like T cells remains to be examined.

Induction of NK-related receptors on T cells may not be surprising since T cells and NK cells originate from a common lymphoid progenitor. We have shown that NK cells have an abundance of untranslated, but re-arranged, *TCRαβ* mRNA with sequences identical to those seen in T cells (130). Thus, inducibility of NK-related receptors in senescent CD28^{null} NK-like T cells is consistent with functional plasticity of T cells (131–133). Although the intricacies of T cell plasticity are still being investigated, such plasticity re-directs the elaboration of effector activities to ensure a vigorous immunity. In old age, signaling of effector activities of NK-like T cells through NK-related receptors is an adaptation of the aging immune system. Such adaptation is a way to maintain immune homeostasis despite the inefficiency of classical TCR signaling and the contraction of diversity of the repertoire of clonotypic TCRs. NK-like T cells are highly resistant to cell death (12) and may represent Darwin's "fittest" lymphocytes that contribute to immune function into old age.

Cell senescence is undoubtedly a characteristic of old organisms, and it contributes to age-related malfunction in various tissues/organs (44, 134, 135). However, cell senescence also has physiologic benefits. Among these is its role in tumor suppression (134, 136, 137). Cell senescence also plays a role in tissue repair (138), such as in the prevention of fibrosis in liver, skin, kidney, and heart, and in the prevention of atherosclerosis and pulmonary hypertension (139). In addition, there is also programed cell senescence, which is an essential component of embryogenesis (140–142). Along these lines, the age-dependent emergence of functionally competent senescent NK-like CD28^{null} T cells represents a significant and beneficial remodeling of the immune repertoire (123).

T cell repertoire remodeling through the *de novo* expression of NK-related receptors along clonal lineages of senescent CD28^{null} T cells is also consistent with age-related functional plasticity in certain organ systems. For example, there is age-related structural and functional decline in the central nervous system that leads to varying degrees of cognitive impairment, such as dementia and Alzheimer's disease. There is heritability of high cognitive function into old age (143, 144). The roles of specific genes or gene polymorphisms, and epigenetic programs have been reported (114, 115, 119, 145–150). But the apparent "default" trajectory of age-related cognitive decline may be altered by physical activity, inclusive of regimented exercise, strength training, or usual activity such as walking. This has been best illustrated by improvement of various aspects of cognitive function, including memory and learning, among older adults engaged in regular physical activity (151–158). Functional brain imaging show extraordinary brain networks of neurocognitive performance following physical activity (159–161). In experimental animals, physical activity elicits an array of genes, along with epigenetic changes, associated with improvement in neurobehavioral performance (162–165). While the mechanisms underlying the improvement of brain/cognitive function with physical activity need to be examined further, aging of the brain is undoubtedly amenable to modulation.

Similarly, aging leads to a decline skeletal muscle function, including an age-related inefficiency of muscle mitochondria. Yet, the aging skeletal muscle is functionally plastic. Whereas certain gene polymorphisms have been implicated to maintain muscle function with age (166), physical activity has been shown to improve muscle and mitochondrial function among older adults (167–170). An important component of physical activity-induced improvement of function of the aging muscle is the equally plastic satellite cells that maintain muscle organization (171, 172). Clearly, certain physiologic systems including immune cells are functionally plastic, a property that may be exploited to maintain, if not improve, functional performance in old age.

NK-LIKE T CELL SUBSETS ARE BIOINDICATORS OF SUCCESSFUL AGING AND LONGEVITY

As described previously, older adults are highly heterogeneous, with varying health phenotypes and life expectancy. An improved understanding of this heterogeneity has been facilitated by objective measurements of physical and cognitive function. Such measurements have led to better stratification of elders; from

frail residents of long-term care facilities, to successfully aging community dwellers (16, 31, 153, 173–177). Thus, we have been proponents for the integration of immunity with other domains of function (32).

Integration of immunity to other physiologic systems may be best illustrated by our cross sectional study of the All Stars cohort (70) of the survivors from the Cardiovascular Health Study, a multicenter long-term study of aging (16, 178, 179). Categorization of elders was based on cognition scores (3MS), measured by the average of three tests using the modified minimental examination (180), and self report of difficulty in performing activities of daily living (ADL), namely, dressing, toileting, transferring, eating, and bathing (181). High functioning (or "unimpaired") was defined as 3MS score >80 and ADL = 0. The data showed that the stimulatory NK-related receptors CD16, CD56, and NKG2D in all T cell subsets were the most prominent cellular components of the immune signature of the high functioning group as determined by factor analysis. In contrast, the inhibitory NK-related receptors NKG2A, CD158a, and CD158e comprised the cell signature of the functionally impaired. In line with these fingerprints, logistic regression analysis of the same dataset showed CD56 and CD16 expression was significant predictors of high functional performance. In contrast, NKG2A and CD158a were negative predictors. More importantly, $CD28^{null}$ T cells in the CD4 but not in the CD8, compartment expressing these four NK-related receptors were the cell subset predictor of high cognitive/physical functioning.

Another way to illustrate the relationship between NK-like $CD28^{null}$ T cells and physical/cognitive functioning is shown in **Figure 2** with a three dimensional plot for CD16 or CD56 expression levels (measured as GMFI, geometric mean fluorescence intensity), 3MS cognition score, and gait speed. The latter measure of physical function was determined by a 4-m walk test that has been standardized/validated from various cohort studies (16, 176). The data show a clear segregation between the high functioning and functionally impaired elders. This is surprising given that "impaired" and "unimpaired" categories in this graphical illustration are very loosely defined by ADL $\geq$ 1 and ADL = 0, respectively. Therefore, it will be of significant interest to determine if this three-way relationship between subsets of NK-like T cells, physical function, and cognitive ability translates into vigorous immune defense. In addition, the underlying mechanistic link(s) between these three physiologic systems will be instructive about integrative physiology of successful aging.

NK-LIKE T CELLS IN YOUNG PERSONS WITH CHRONIC DISEASES: A CASE FOR ANTAGONISTIC PLEIOTROPY

NK-like $CD28^{null}$ T cells represent a beneficial remodeling of the T cell repertoire with aging. Paradoxically, similar cells have also been found among young patients with chronic immune-mediated diseases in an age-disproportionate manner. We have shown the infiltration of $CD56^{+}$ $CD28^{null}$ $CD4^{+}$ T cells in extra-articular lesions in rheumatoid arthritis (182). Inflammatory $CD56^{+}$ T cells have been reported in coronary artery disease, asthma, ulcerative colitis, and chronic hepatitis C disease (183–186). $NKG2D^{+}$ $CD28^{null}$ T cells have some tumor-promoting activity in experimental settings (187, 188) and as inflammatory mediators in Wegener's granulomatosis, rheumatoid arthritis, juvenile-onset systemic lupus erythematosus, and celiac disease (189–192).

Many of these diseases have characteristic systemic upregulation of TNFα (193). We have shown that TNFα can directly block the *CD28* transcriptional initiator (65, 194). In a TNFα-rich environment, such as in the case of rheumatoid arthritis, we found that anti-TNF therapy prevents the TNFα-induced loss of CD28 on the residual $CD28^{+}$ $CD8^{+}$ and $CD4^{+}$ T cells, but the numbers of $CD28^{null}$ T cells remain the same (194). Whether or not TNFα induces the gain NK-related receptors has not yet been examined.

Interestingly, $CD56^{+}$/$NKG2D^{+}$ T cells also have beneficial effects in disease settings. Regulatory $CD56^{+}$ $CD28^{null}$ $CD8^{+}$ T cells and $NKG2D^{+}$ T cells have been reported in rheumatoid arthritis and in juvenile-onset systemic lupus erythematosus, respectively (195, 196). Similar NK-like T cell subsets appear to be normal components of regional host defense in the gut. They may have auxiliary antitumor effect and have been associated with antiviral immunity in the setting of allergies and chronic hepatitis B disease (197–200).

Such age-disproportionate emergence of senescent $CD28^{null}$ NK-like T cells supports the provocative idea that premature senescence of T cells is a critical factor in the pathogenesis and clinical prognosis of chronic diseases of the young (201). These apparent beneficial and detrimental effects of certain NK-like T cell subsets among young patients, and the beneficial effects of similar cells during aging as described above, are consistent with the evolutionary concept of antagonistic pleiotropy (202). This concept posits that genes and biological pathways that are beneficial in the young may be detrimental in the old, and vice-versa. Therefore, a scientific challenge is to determine conditions in disease states of the young where $CD28^{null}$ NK-like T cells might exert a pathogenic effect. It will be of similar interest to determine what drives the accumulation of beneficial senescent $CD28^{null}$ NK-like T cells during the aging process.

CONCLUSION: THE CHALLENGE OF HARNESSING BENEFITS OF $CD28^{null}$ NK-LIKE T CELLS

The expression of NK-related receptors along clonal lineages of $CD28^{null}$ T cells with aging clearly represents a reshaping or remodeling of the immune repertoire. T cell signaling through these receptors independent of the TCR also illustrates the emerging theme that cell senescence may not necessarily be synonymous with dysfunction. One scientific challenge is to determine what drives the induction of diversity of expression of NK-related receptors on T cells with advancing age. Another is to determine whether the TCR-independent effector function of NK-like T cells translates into vigorous immune defense

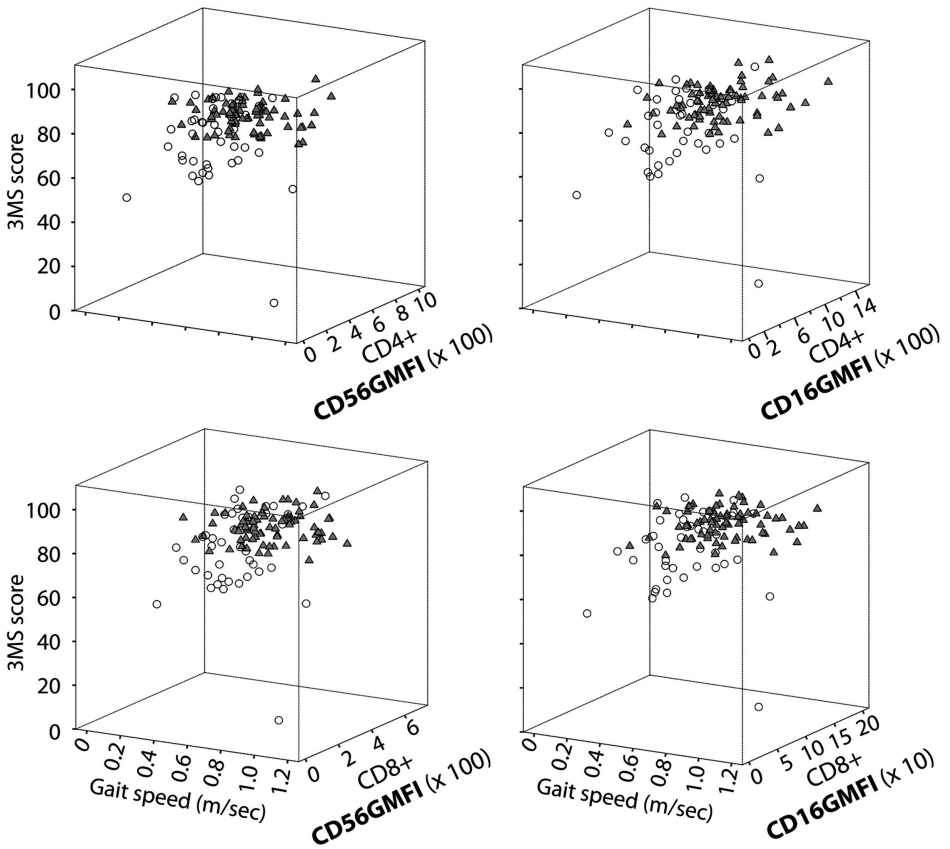

FIGURE 2 | **NK-like T cells are linked to high cognitive and physical function**. Data shown are 3D scatter plot summaries from the re-analyses of our data from the All Stars cohort of the Cardiovascular Health Study (70). CD16 and CD56 expression on $CD4^+$ $CD28^{null}$ and $CD8^+$ $CD28^{null}$ T cells are expressed as GMFI, which was determined by multicolor flow cytometry. Older adults were grouped as unimpaired (*solid triangles*) or impaired (*open circles*) based on a simple criterion of ADL = 0 and ADL > 1, respectively. Measurements of 3MS cognition score and gait speed and ADL scoring are as described in the text.

and/or immune surveillance in late life. A corollary interest is a possible dual functionality of these T cells, namely, their ability to trigger a classic TCR-driven response, while triggering a complementary innate TCR-independent response mediated through the particular NK-receptor(s) they express. Plausibility of this dual function has been shown experimentally for the interaction between tumor cells and particular NK-like $CD8^+$ T cell lines *in vitro* (203). An equal challenge is to elucidate the paradoxical age-disproportionate accumulation of NK-like T cells in disease states. Whether they represent cells involved in tissue repair or if they are true pathogenic effectors will be instructive into harnessing or dampening their effector function in disease settings. During the aging process, the most significant challenge is to determine how and why particular subsets of NK-like $CD28^{null}$ T cells are closely linked to physical performance and cognitive ability. Dissecting these mechanisms will depend on the analyses of defined populations of the elderly, rather than continuing with the usual young-versus-old comparisons.

AUTHOR CONTRIBUTIONS

JM, PG, and AV drafted and edited the manuscript. JM and PG generated the figures. AV secured funding.

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T Cell Subpopulations in Healthy Elderly and Lung Cancer Patients: Insights from Cuban Studies

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The senescence of the immune system and the risk of cancer increase with aging. Age itself entails changes in the immune system, which are related to a decrease in thymic output of naïve lymphocytes, an accumulation of chronic antigenic load, notably chronic viral infections such as cytomegalovirus (CMV), and replicative senescence of lymphocytes. These changes could eventually contribute to cancer risk and affect the response to cancer treatment. However, several confounding factors make it difficult to draw a picture of causal relationships. Studies in diverse human populations could contribute to clarify these complex relationships. Here, we summarize the current knowledge about the senescence of the T cells, the relationship with CMV infection, cancer, and cancer treatment. We also review the results of a series of studies performed in Cuba whose population is characterized by the unusual combination of long life expectancy and high antigenic load, including high seroprevalence of CMV, typical of tropical countries. Although immunosenescence affects almost all components and functions of the immune response, its most salient feature is a decrease in numbers and proportions of naïve $CD8^+$ T lymphocytes and an accretion of terminally differentiated $CD8^+$ T lymphocytes. These features were confirmed by the Cuban studies, but interestingly a clear gender effect also appeared. Moreover, as aging is a global phenomenon, a fast increase in elderly with malignancies is expected; therefore, the evaluation of patient's immune status would support the decision of treating them with immunotherapy and predict the efficacy of such treatments, thereby improving benefits for the patients.

Keywords: CD8 T cells, late-stage differentiated CD8 T cells, non-small cell lung cancer, cancer vaccine, CMV

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INTRODUCTION

Aging is related to changes in innate and adaptive immune system. Those age-related changes could be associated with susceptibility to infectious diseases, Alzheimer's disease, autoimmunity, osteoporosis, and cancer (1).

However, several potentially confounding factors make it difficult to draw a clear picture of causal relationships. For example, the risk of cancer increases with age and cancer itself. Cancer treatment could also influence the immune system and chronic infections such as cytomegalovirus (CMV) could drive immunosenescence. These processes occur simultaneously; nevertheless, it is not yet determined in what magnitude they are causally related (2).

The changes most consistently found in immunosenescence studies pertain to $CD8^+$ T cells. It is well documented that with decrease in age naïve $CD8^+$ T cells, highly differentiated $CD8^+$ T cells lacking CD28 accumulate and those $CD8^+CD28^-$ T cells upregulate the expression of CD45RA. Additionally, these cells show signs of replicative senescence such as a decreased proliferation ability, shortened telomeres, impairment of telomerase activity, and upregulation of CD57 (3, 4). These facts have been frequently associated with chronic CMV infection (5).

The relationship between these changes and a susceptibility to disease was first documented in the Swedish octogenarian study (OCTO-immune). This study defined the concept of "immune risk profile" (IRP). The IRP was described as a decrease in number and frequency of B cells, an increased count of $CD8^+$ memory T cells, a CD4/CD8 ratio of less than 1, and a rise of $CD8^+CD28^-$ among other late-stage differentiated T cells (6). Additionally, most of these late-stage differentiated cells express CD57 (7). The IRP was also associated with high serum concentrations of pro-inflammatory cytokines and seropositivity to CMV (6, 8).

High prevalence of CMV is found worldwide; however, it fluctuates depending on the region (9). The CMV infection frequently starts during adolescence and persists throughout life. The expansion of $CD8^+CD28^-$ T cells upregulating CD57 has been associated with CMV infection. It is considered one of the main causes of immunosenescence (8).

A possible connection between the high prevalence of CMV infection in tropical countries and some sociocultural behaviors, which contribute to CMV transmission in early stages of life, has been postulated (10). Our group confirmed a high seroprevalence of CMV infection in healthy Cubans from an early age (4).

In the present paper, the current knowledge on the dynamics of T cell subpopulations during aging is reviewed, as well as the relationship with CMV infection, cancer, and cancer treatment. The results of several studies carried out in Cuba are also interpreted. As Cuban population presents an unusual combination of a long life expectancy and high antigenic load of a tropical country, it is a kind of natural experiment, which could show novel aspects in the relationships between immunosenescence and chronic non-communicable diseases.

THE INCREASED PROPORTION OF LATE-DIFFERENTIATED CD8 T CELLS IN HEALTHY CUBANS IS INFLUENCED BY AGE AND GENDER

Long-lasting antigenic stimulation causes the progressive increase of late-stage differentiated, oligoclonal T cells, mainly, but not exclusively, within the $CD8^+$ T cell compartment. Increasing evidence demonstrate that the $CD8^+CD28^-$ and $CD8^+CD57^+$ T cell populations play an essential role in innumerable diseases or chronic inflammation-related conditions, associated with chronic immune stimulation such as cancer, chronic intracellular infections, chronic pulmonary diseases, autoimmune diseases, and allogeneic transplantation (11).

Older individuals tend to exhibit abundance of late-stage differentiated memory T cells. CD57 is a receptor expressed on $CD8^+$ and $CD4^+$ T cells in late stages of differentiation (12). Late-differentiated T cells are characterized by the expression of CD45RO, reduced or almost undetectable expression of costimulatory molecules CD27 and CD28, and chemokine receptor CCR7. The re-expression of CD45RA is also characteristic (13).

Previous reports confirmed that $CD57^+CD8^+$ T cells can be defined as "replicatively senescent cells," although these cells are not "functionally exhausted" (13). The progressive decrease of T cell function as a consequence of a chronically high antigen load is described as part of the phenomenon of exhaustion (14). Nevertheless, senescent $CD57^+CD8^+$ T cells are able to secrete TNF-α and IFN-γ upon encounter with antigen (15).

$CD8+CD28^-$ T cells are end-stage cells that have lost the expression of CD28. Lin and colleagues showed that they had the lowest telomerase activity among memory and highly differentiated $CD8^+$ T cell subsets. Consistent with this finding, those researchers also demonstrated that $CD8^+CD28^-$ T cells had "the shortest mean telomere length" among the studied groups of memory T cells (16).

Prior studies suggest that the replicative capacity of $CD8^+CD28^-$ T cells in response to antigen stimulation is significantly reduced and the telomere length is shorter in comparison with $CD8^+CD28^+$ T cells (17). High percentages of $CD8^+CD28^-$ T cells are related to reduced response to vaccination (18) and have been associated with mortality in a cohort of elderly Swedish (19).

A study in healthy Cubans showed an effect of gender in the dynamics of T cell subpopulations during aging. Although the proportion of late-differentiated $CD8^+CD28^-$ and $CD8^+CD57^+$ T cells increased with age, it was only statistically significant in males. Cuban women preserve the proportion of $CD8^+CD28^-$ and $CD8^+CD57^+$ T cells practically constant at all ages. Our group also reported other changes with age within the $CD8^+$ T cell subset. In this occasion, an increased frequency of terminally differentiated $CD8^+CD45RA^+CD28^-$ T cells was found in females as they aged, while males showed higher frequency of these cells from youth. By contrast, within $CD4^+$ T lymphocytes, terminally differentiated $CD4^+CD45RA^+CD28^-$ T cells showed an age-associated increase in both sexes, though higher proportions were found in males (4).

The detection of differences concerning the structure and function of the immune system in males and females has been described (20, 21). In addition to hormones, genetic factors can determine the differences in the immune response between males and females. The fact that some genes in the X chromosome are involved in immunity has been addressed (21). It has also been proposed that estrogens improve humoral immunity, whereas androgens and progesterone have a tendency to hamper it (22).

Gender differences in the immune system have been evidenced also in epidemiological studies showing higher incidence of autoimmune diseases in females and higher rate of chronic inflammatory illness such as atherosclerosis-related diseases in males. Nevertheless, the interaction among hormones, genetics, inflammation, and immune system presents a complex scenario that must be more intensively studied (4).

The influence of gender in immunosenescence also appeared in the Berlin Aging Study II, which reported gender-related differences concerning the consequences of age and CMV infection

on CD4 and CD8 T cells. This study reported that older men showed higher frequencies of late-differentiated $CD8^+CD57^+$ T cells and concluded that in elderly men, the "CMV-associated senescence of T cells" was more pronounced than in elderly women (23).

The Berlin study also showed a strong effect of CMV infection in the appearance of $CD45RA^+CCR7^-CD27^-CD28^-$ terminally differentiated T cells (so-called TEMRA). They observed a significantly lower proportion of $CD4^+CD45RA^+CCR7^-CD27^-CD28^-$ T cells in CMV-negative individuals than in CMV-positive individuals, regardless of gender and age. Concerning the frequency of $CD8^+$ T cells, age was observed to have a significant influence, but here too, it was only significant in subjects with demonstrated CMV infection. Therefore, frequency of terminally differentiated T cells was significantly higher in CMV-positive elderly individuals than it was in CMV-negative elderly, notwithstanding gender (23).

Cytomegalovirus is a common herpes virus affecting the 60–90% of the global population. The prevalence of infected individuals increases with age. It is expected that 90% of individuals could be infected nearby the 90 decade of life in contrast with the evidence of 40–60% of individuals in the middle age population (24). The OCTO study reported that the prevalence of subjects with CMV–IgG antibodies in individuals older than 80 years was around 90%, while in middle-aged individuals, it was relatively consistent at 67% (6). Interestingly, our group reported a high seroprevalence of CMV seropositivity greater than 80% in Cuban healthy population from young ages (4).

Persistent infection with viruses such us CMV can augment the accumulation of senescent $CD4^+$ and $CD8^+$ T cells, identified as $CD27^-CD28^-CD45RA^+KLRG1^+$ and $CD57^+$, compared to age-matched seronegative individuals (25). The research in this area has been predominantly focused on $CD8^+$ T cells, which display decreased naive populations and increased memory subset distribution consistent with a more memory/late effector cell profile. This is accompanied by changes in function such as reduced proliferative capacity, especially in $CD57^+$ T cells, and increased cytotoxic and secretory functions (26, 27).

However, changes in T cell subpopulations are also evident in $CD4^+$ T lymphocytes. A recent work described the significant increment in the percentages of $CD4^+CD57^+$ T cells in young CMV-positive individuals, compared with young CMV-negative individuals. They showed that $CD4^+$ T cells that coexpress CD57 and CD154 are only present in CMV-positive subjects and are considered a very polyfunctional $CD4^+$ subset (28). This group had previously revealed an increase in $CD8^+CD57^+$ T cells in CMV-positive young subjects (29).

The CMV-IgG seropositivity was determined in healthy Cubans of all ages in a study conducted by Garcia and colleagues. The general seroprevalence of CMV seropositivity was 90%. More than 90% of elderly individuals had antibodies against CMV, notwithstanding their gender. Nevertheless, young males (93.3%) had higher seroprevalence than young females (73.6%) (4).

In our analysis, the higher percentage of $CD45RA^+CD28^-$ T cells within the $CD4^+$ and $CD8^+$ subsets described in Cuban males, but not in females, during young ages can be explained by the differential effect of gender and age in the thymic output (4). Furthermore, since androgens and testosterone have higher association with severe thymus involution than female hormones, young males could have less protected immune system than females. This combined with exposure to high antigenic loads since early ages could have driven immunosenescence in young males (4, 30).

The thymic involution in elderly people induces a reduction of naive T cells in the periphery, regardless of CMV infection. Nonetheless, during persistent CMV infection, the memory T cells frequency is higher, possibly because of the latency of CMV, which exerts a persistent stimulation on the immune system in order to control the virus (24, 26). Additionally, CMV reactivation may occur more often in older people (31). From this point, an impairment of the immune system would hamper its capacity to control the CMV infection; therefore, a reactivation of the CMV would lead to a long-lasting antigen stimulation and accelerate the accumulation of $CD28^-$ T cells as well as the emergence of the phenomenon of immunosenescence, functioning as a closed loop (24).

TREATMENT WITH PLATINUM-BASED CHEMOTHERAPY ENTAILS DIFFERENT PATTERNS OF TERMINALLY DIFFERENTIATED CD8 T CELLS IN NSCLC PATIENTS

Around 70% of cancer-related deaths and 60% of new cancer diagnosis occur in patients older than 65 years (1). Moreover, as aging is a global phenomenon, a rapid increase in elderly with malignancies is expected (32).

A study in cancer patients (respiratory, digestive, reproductive, head, and neck) showed an expansion of $CD8^+CD28^-$ T cells in heavy chemo-treated patients compared with healthy volunteers and treatment-naive patients (33). Another research in patients with various forms of lung cancer receiving chemotherapy reported higher proportions of $CD28^-CD57^+$ cells, thereby highlighting the most pronounced changes in lung cancer patients with stage IV of the disease (34).

Recently, high proportions of $CD4^+CD28^-$ and $CD4^+CD57^+$ T cells have been reported in CMV-positive glioblastoma patients. Additionally, these researchers described short survival in glioblastoma patients with high proportions of $CD4^+CD28^-$ and $CD4^+CD57^+CD28$ null T cells, thereby suggesting an association between those immunosenescence markers and survival in CMV-positive glioblastoma patients (35).

Our group evaluated the presence of the CD28 receptor on $CD4^+$ and $CD8^+$ T cells in a cohort of Cuban advanced NSCLC patients, before and after administration of first-line platinum-based chemotherapy. We found that the proportion of $CD4^+CD28^-$ T cells significantly increased in NSCLC patients after treatment with platinum-based chemotherapy, compared with healthy volunteers and with cancer patients without chemotherapy. Healthy volunteers and cancer patients without chemotherapy had low proportions of $CD8^+CD28^-$ T cells.

Cancer patients treated with standard front-line chemotherapy showed the highest proportions of $CD8^+CD28^-$ T cells (36).

In addition, our group investigated the frequency of $CD8^+CD57^+$ T cells and $CD45RA^+CD28^-$ on $CD4^+$ and $CD8^+$ T cells in Cuban NSCLC patients after front-line platinum-based chemotherapy. We showed that the frequency of $CD8^+CD57^+$, $CD4^+CD45^+CD28^-$, and $CD8^+CD45^+CD28^-$ T cells remained unchanged, regardless of the presence of cancer itself or the chemotherapy treatment.

Based on the high prevalence of CMV infection in Cubans and on previous findings in healthy elderly (4), we hypothesized that notwithstanding cancer disease or chemotherapy, aging and possibly chronic CMV infection could be the main causes for the increase of $CD45RA^+CD28^-$ T cells (36). In our opinion, platinum-based chemotherapy probably causes only the increase of $CD28^-$ T cell subpopulations within $CD4^+$ and $CD8^+$ subsets. Otherwise, changes in the frequency of $CD4^+$ or $CD8^+CD45RA^+CD28^-$ and $CD57^+CD8^+$ T cells seems not to be related with cancer disease or chemotherapy.

Although consensus about necessary chronic antigen stimulation, especially CMV, to cause immunosenescence is under constant discussion, maintaining CMV reactivations under control requires a huge effort from the immune system. This virus could be responsible for the functional impairment of many cell types from innate and adaptive immune systems. Besides affecting the immunosurveillance, this virus could also contribute to the pathogenesis of some inflammatory diseases and even cancer (24).

$CD8^+CD28^-$ T CELLS AND CD4/CD8 RATIO AS PREDICTIVE BIOMARKERS OF EFFICACY OF THERAPEUTIC VACCINATION WITH THE EPIDERMAL GROWTH FACTOR (EGF)-BASED VACCINE CIMAvax-EGF

The suppression induced by tumor disease and by standard therapies such as chemotherapy and radiation can influence detrimentally the immune system of cancer patients. Nowadays, the assessment of a patient's immune status represents a valuable tool for determine patients for undergoing immunotherapy (37, 38).

Biomarkers are becoming more necessary in order to select patients who could benefit from therapies, either in the initial phase of the disease or in advanced cancer stages. In such cases, the definition of personalized treatments in tumor disease could lead to an improvement of therapeutic success (39).

CIMAvax-EGF is a therapeutic cancer vaccine developed to generate specific humoral response against the EGF (40). More than 4,000 advanced NSCLC patients have been treated with the CIMAvax-EGF vaccine, which is safe and immunogenic and have proved its efficacy (41).

As previous results published by our Institute showed, a relation between the magnitude of specific anti-EGF antibody response and the clinical outcomes of vaccinated patients has been demonstrated when using CIMAvax-EGF as switch maintenance therapy after platinum-based first-line chemotherapy in NSCLC patients. Young patients showed the best clinical results (42, 43). These results suggest that the clinical benefit in CIMAvax-EGF vaccinated NSCLC patients goes together with the development of a good specific humoral response (36).

In a recent article, our group proposed the frequencies of $CD8^+CD28^-$ T cells and the CD4/CD8 ratio as possible predictive biomarkers for the CIMAvax-EGF efficacy. Consequently, NSCLC patients with a proportion of $CD8^+CD28^-$ T cells of less than 24% and a CD4/CD8 ratio >2 determined after front-line standard chemotherapy and prior to vaccination with CIMAvax-EGF achieved a median survival superior by almost 20 months to that of vaccinated patients with more than 24% of $CD8^+CD28^-$ T cells and a CD4/CD8 ratio <2. These findings emphasize the impact of the immune status on the clinical evolution of CIMAvax-EGF vaccinated NSCLC patients and validate the usefulness of late-stage differentiated $CD8^+$ T cells as predictive biomarkers for the CIMAvax-EGF efficacy (36).

As studies of the senescence of the immune system advance, it can be predicted that markers of the dynamics of lymphocytes and cytokines along individual's lifetime will be increasingly used as prognostic factors and treatment predictors in several diseases. In contrast to genetic markers, which are mainly endogenous individual traits, markers of immunosenescence evolve under extrinsic environmental influences, which obviously vary among diverse human groups. Thus, findings are difficult to extrapolate from country to country. For this reason, specific studies in diverse populations are needed to better asses the timelines of immunosenescence and the influence of chronic antigenic loads and gender. It has been predicted that different forms of immunotherapy will become part of the main therapeutic strategy in an increasing fraction of cancer patients in the near future. This scenario will make specific studies of immunosenescence mandatory.

AUTHOR CONTRIBUTIONS

DS, AL, and BG have overall responsibility for writing the paper.

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Adaptive Memory of Human NK-like CD8$^+$ T-Cells to Aging, and Viral and Tumor Antigens

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Human natural killer (NK)-like CD8$^+$ T-cells are singular T-cells that express both T and NK cell markers such as CD56; their frequencies depend on their differentiation and activation during their lifetime. There is evidence of the presence of these innate CD8$^+$ T-cells in the human umbilical cord, highlighting the necessity of investigating whether the NK-like CD8$^+$ T-cells arise in the early stages of life (gestation). Based on the presence of cell surface markers, these cells have also been referred to as CD8$^+$KIR$^+$ T-cells, innate CD8$^+$ T-cells, CD8$^+$CD28$^-$KIR$^+$ T-cells or NKT-like CD8$^+$CD56$^+$ cells. However, the functional and co-signaling significance of these NK cell receptors on NK-like CD8$^+$ T-cells is less clear. Also, the diverse array of costimulatory and co-inhibitory receptors are spatially and temporally regulated and may have distinct overlapping functions on NK-like CD8$^+$ T-cell priming, activation, differentiation, and memory responses associated with different cell phenotypes. Currently, there is no consensus regarding the functional properties and phenotypic characterization of human NK-like CD8$^+$ T-cells. Environmental factors, such as aging, autoimmunity, inflammation, viral antigen re-exposure, or the presence of persistent tumor antigens have been shown to allow differentiation ("adaptation") of the NK-like CD8$^+$ T-cells; the elucidation of this differentiation process and a greater understanding of the characteristics of these cells could be important for their eventual in potential therapeutic applications aimed at improving protective immunity. This review will attempt to elucidate an understanding of the characteristics of these cells with the goal toward their eventual use in potential therapeutic applications aimed at improving protective immunity.

Keywords: NK-like CD8$^+$ T-cells, memory, T-cell differentiation, immunosenescence, aging, CMV, natural killer receptors, CD56

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INTRODUCTION

T lymphocytes derive their name from their site of maturation in the thymus. In particular, T cytotoxic (Tc) cells that express CD8 are activated upon interaction with an MHC-class I complex on the surface of an altered-self cell (e.g., virus-infected cell or tumor cell) in the presence of appropriate cytokines. T-cell co-signaling is largely context dependent and relies on a diverse array of costimulatory and co-inhibitory receptors spatiotemporally regulated, which may have distinct or overlapping functions in T-cell priming, activation, differentiation, and memory responses (1). The total cytotoxic

CD8+ T-cell pool is exposed to different microenvironmental stimuli (both TcR dependent and independent) and the resulting phenotype and cytokine secretion will determine an individual T-cell or T-cell clone's effector or regulating functional capacities, including tissue residence/homing and organ homeostasis (2).

In addition to CD8+ T lymphocytes, natural killer (NK) cells have a crucial role in the recognition and killing of virus-infected/tumor cells, but unlike CD8+ T-cells, they use a repertoire of germ-line encoded inhibitory/activating receptors that recognize "missing self"/"altered-self" antigens on the target cells leading to cytotoxicity and cytokine production (3). These NK cell receptors (NKRs) are also expressed on certain subsets of T-cells. One example is NKR-CD56, which has been found to be elevated in both peripheral blood cells and in tumor-infiltrating lymphocytes in patients with colorectal cancer (4). In many clinical circumstances, the expression of different NKRs on T-cells is associated with prolonged antigen stimulation, suggesting that these receptors play a crucial role in the homeostasis of antigen-experienced T-cells.

Cumulative evidence supports the existence of T-cell subsets, with characteristics that bridge innate and adaptive immunity, which are relevant in inflammation and viral and tumor surveillance, and which could have a role in the pathogenesis of autoimmune diseases. These NKR-expressing cytotoxic T lymphocytes (CTL) have been termed NK T (NKT) cells. Thus, NKT cells are naturally occurring, although rare, T-cells that express both T and NK cell receptors (5). However, there is some confusion with the use of the term "NKT-cell." On one hand, CD1d-restricted cells, which have a semi-invariant TcR, are frequently called NKT-cells or invariant NKT (iNKT) cells; on the other hand, highly specialized effector memory CD8+ T-cells expressing NKRs are also referred as NKT-like cells. Therefore, to avoid confusion, we will call the later, NK-like CD8+ T-cells.

Natural killer-like CD8+ T-cell differentiation occurs after the induction of transduction signals that activate/inhibit the expression of certain CD8+ T-cells genes, determining the activation state, proliferation, and differentiation (6). Indeed, prolonged antigen stimulation may induce changes in the CD8+ T-cell receptor repertoire leading to the expression of NKRs; and chronic antigen stimulation of T cells also leads to other phenotypic changes such as the loss of costimulatory molecules (e.g., CD28) (5). Usually, CD8+ T-cell memory subsets display specific responses based on the expression of killer cell immunoglobulin-like receptors (KIRs) used to distinguish unhealthy cellular targets from the healthy host cells (7, 8). However, high antigen concentrations can bypass the KIR-mediated inhibition of T-cell activation. Dynamic KIR expression may mediate T-cell tolerance to self-antigens by down regulating self-reactive T-cells (9). Nevertheless, the functional significance of the inhibitory or activating NKRs on NK-like CD8+ T-cells is still unclear.

Natural killer-like CD8+ T-cells have been described using different names, for example: CD56+CD8+ NKT-like cells (10), CD28−KIR+ CD8+ T-cells (5), KIR+CD8+ T-cells—particularly those expressing NKG2A—(11), or the general term innate CD8+ T-cells—since NKR+ αβT-cells likely represent immune effector cells that are capable of combining innate and adaptive functions (12). **Table 1** summarizes the most relevant information regarding the characterization of NK-like CD8+ T-cells.

In both the human umbilical cord and in healthy adults, an "innate/memory-like" CD8+ T-cell subset that expresses KIR and NKG2A has been described. These cells were EOMES^{hi}, exhibited potent antigen-independent cytotoxic activity, and

TABLE 1 | Characterization of NK-like CD8+ T-cells.

Study	Cell name	Specie	Biomarkers	Result	Reference
The expression of NK receptors in PBL from healthy and melanoma patients	CD28− cytolytic effector T cells	Human	CD28− preferential alpha/beta TcR+ killer cell immunoglobulin-like receptor (KIR)+	The percentages of NK receptor-positive (p58.1, p58.2, p70, p140, ILT2, NKRP1A, ZIN176, CD94, and CD94/NKG2A) T cells (NKT cells) varied more strongly between melanoma patients	Speiser et al. (5)
Changes in the T cell pool caused by CMV infection contribute to immunosenescence	CD8+ natural killer (NK) T-like cells	Human	CD56+	NKT-like cell percentage increases with the combination of both CMV and age	Hassouneh et al. (10)
Eomesodermin-expressing innate-like CD8+ KIR/NKG2A+ T cells in human adults	KIR/NKG2A+ CD8+ T cells	Human	KIR+ (NKG2A) innate/memory phenotype	Increased Eomes expression, prompt IFN-gamma production in response to innate-like stimulation by IL-12 + IL-18	Jacomet et al. (11)
Review: expansions of NK-like alpha/beta TcR T cells with chronologic aging	NK-like alpha/beta TcR T cells	Human	NK cell receptors on aged alpha/beta TcR cells	NKR expression on T cells is physiologically programed rather than a random event of the aging process	Vallejo et al. (12)
Regulation of the immune response through killing antigen-bearing DCs	CD8+ NKT-like cells	Mice	TcR beta CD3 NK1.1+ CD49b+ NKG2D+	Secretion of high levels of IFN-gamma, but not IL-4	Wang et al. (13)
T-cell responses and protective immunity	Memory-like effector NKT cells	Mice	CD44+ CD62L−	Coadministration of alpha-GalCer analog and TLR4 agonist activates memory-like effector NKT cells	Coelho-Dos-Reis et al. (14)

produced IFN-gamma in response to IL-12 + IL-18 (11). The differentiation of these cells, similar to that of the overall pool of CD8+ T-cells, can be influenced by aging. Specifically, age has been associated with increased susceptibility to infections and inflammatory diseases (15), cancer, and autoimmunity (16). The differentiation of human NK-like CD8+ T-cells is initiated after viral/tumor antigen priming and may be influenced by other factors such as aging, autoimmunity, or inflammation. These cytotoxic CD8+ T-cells, when activated, implement a differential expression of NKRs, leading to memory and migration responses. According to the expression of the CD8 marker on their surface, these cells are classified as bright or dim and display different functional properties (**Figure 1A**). In this context, we reviewed the literature regarding NK-like CD8+ T-cells and their phenotypic characterization associated with viral infection, immunosenescence, and diseases.

PHENOTYPIC CHARACTERIZATION OF NK-LIKE CD8+ T-CELLS

Immunological memory is the ability of the immune system to respond more rapidly and effectively to previously encountered pathogens. This is a classical feature of adaptive immunity, which is derived from unique patterns of gene expression. A faster and stronger transcription of previously activated genes occurs in memory T-cells compared with naïve cells. This ability to remember past transcriptional responses is termed "adaptive transcriptional memory" (18). After acute infections, CD8+ T-cell memory differentiation leads to the generation of functionally distinct populations, with either proliferative potential or cytotoxic effector functions, that recirculate into lymphoid tissues or remain tissue-resident (6). This phenomenon depends on the expression of several receptors, including the C-C chemokine receptor 7 (CCR7) and CD45RA, which have been used to discriminate naïve (*N*; CD45RA+CCR7+), central memory (T_{CM}; CD45RA−CCR7+), transitional memory (T_{TM}; CD45RA−CCR7−), and terminally differentiated T-cells (T_{EMRA} or T_{TE}: CD45RA+CCR7−) (15). A further classification divides the CCR7−CD8+ T-cell subpopulation into three distinct memory subsets according to the expression of CD45RA: $CD45RA^{null}$, $CD45RA^{dim}$, and $CD45RA^{bright}$ (19).

There are several models of memory CD8+ T-cell differentiation (20) and two new subsets have been recently described: the "stem cell-like memory T-cells" (T_{SCM}) (21) and the "transitional memory (T_{TM}) T-cells" (17). Differentiation of circulating memory CD8+ T-cells starts after antigen challenge and subsequent naïve T-cell activation. The recently described progressive differentiation model proposes that the fate of the T-cells depends on the duration of signaling and the presence or absence of cytokines. Thus, a single naïve cytotoxic T lymphocyte will differentiate gradually to different memory subsets (**Figure 1B**). In consequence, brief antigen stimulation will generate T_{CM} and T_{TM} cells, while sustained stimulation together with the presence of cytokines will lead to T_{EM} and T_{TE} (T_{EMRA}) cells that re-express CD45RA (17, 22–24). Additionally, another type of memory cell has been described, the "resident memory T-cells" (T_{RM}), which are non-recirculating memory T-cells with long-term persistence in epithelial barrier tissues. As shown in **Figure 1B**, it is probable that NK-like CD8+ T-cells emerge from T_{TM}, T_{EM}, or T_{TE} CD8+ T-cell phenotypes; these cells could be circulating or tissue resident.

In addition, some authors suggest that the CD45RO+CD45RA− T-cells comprise diverse memory subsets, including T_{CM}, T_{SCM}, T_{EM}, and T_{RM} subsets, which are heterogeneous in their generation, distribution, and function (24). Thus, T_{RM} cells may persist in the absence of antigens and display several effector functions. Moreover, T_{RM} cells could have evolved to provide rapid immune protection against pathogens. However, autoreactive, aberrantly activated, and malignant T_{RM} cells can contribute to numerous human inflammatory diseases (25).

CD8+ T-cells in lymphoid tissues are naïve, while in mucosal sites, these cells are IFN-gamma producing T_{EM} cells. The T-cell activation marker, CD69, is constitutively expressed by memory T-cells in all tissues, distinguishing them from circulating subsets. T_{RM} cells expressing CD69 are also present in human mucosal and peripheral tissue sites (24). However, the mucosal memory T-cells exhibit additional distinct phenotypic and functional properties (26). In particular, human intrahepatic lymphocytes are rich in CD1d-unrestricted T-cells that co-express NKRs (NK-like CD8+ T-cells), and it is possible that the hepatic epithelial cells and the cytokine milieu play a role in the shaping of these cells. For example, IL-15 is capable of inducing Ag-independent upregulation of NKRs in the CD8+CD56− T-cells. This increased percentage of intrahepatic NK-like CD8+ T-cells could be in part due to a local CD8+ T-cell differentiation (27) and could explain how NK-like CD8+ T-cells differentiate in the human liver.

The distribution and tissue residence of naïve, central and effector memory, and terminal effector subsets is contingent on both their differentiation state and tissue localization. Moreover, T-cells homeostasis, driven by cytokine or TcR-mediated signals, is different in CD4+ or CD8+ T-cell lineages and varies with their differentiation stage and tissue localization (28). In this sense, it is important to investigate NK-like CD8+ T-cells with respect to memory phenotype, functional properties, and long-term differential fates following acute infection or chronic diseases.

NK-LIKE CD8+ T-CELLS IN VIRUS INFECTION AND IMMUNOSENESCENCE

Throughout an individual's lifetime, the memory T-cell percentage undergoes dynamic changes that can be classified into three phases: memory generation during infancy and early childhood, memory homeostasis, which occurs after age 20–25, and immunosenescence (24). This last term refers to the deterioration of the immune system associated with aging, and it is characterized by substantial alterations of the T-lymphocyte subsets (29). An increased expression of NK cell markers on T-cells has been reported to be associated with aging and chronic activation of the immune system, as reflected in the accumulation of effector/senescent T-cells (30). This memory subpopulation is interesting, because the senescence of human T_{TE} CD8+ T-cells is stringently controlled by distinct and reversible cell signaling events (31). Also, there is evidence of a differential regulation of NKR

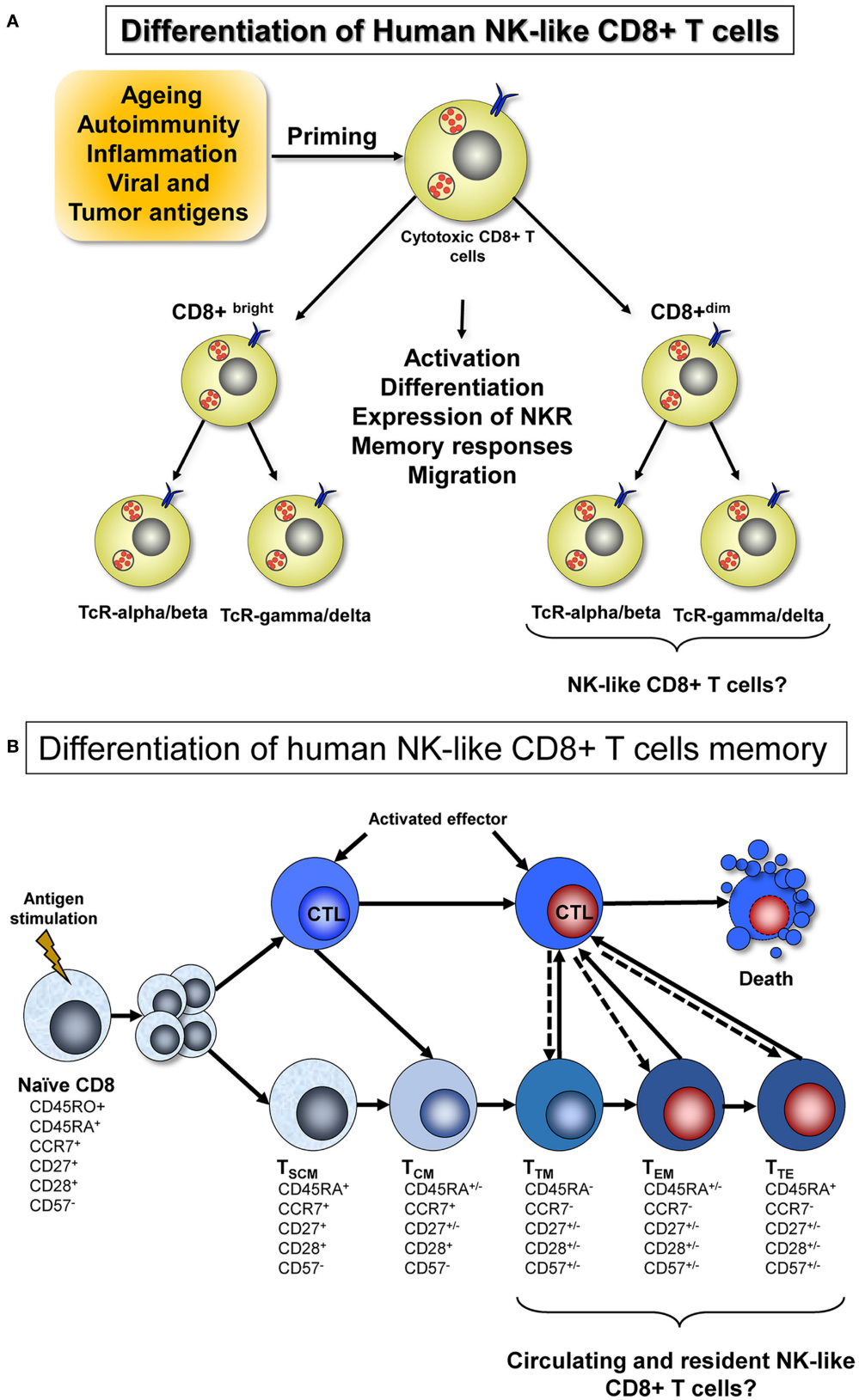

FIGURE 1 | Continued

FIGURE 1 | Continued
(A) Differentiation of human NK-like CD8+ T-cells and expression of TcR. The total pool of cytotoxic CD8+ T-cells is exposed to different TcR-alpha/beta and TcR-gamma/delta dependent or independent microenvironmental stimuli. From this pool originate the NK-like CD8+ T cells, which can be induced by the transduction of signals that activate or inhibit gene expression that, in turn, determines cytokine secretion, effector/regulating functions, migration/tissue retention, activation state, proliferation, and differentiation. Other factors that influence this process are aging, autoimmunity, inflammation, and the presence of viral and tumor antigens. **(B)** Differentiation of human NK-like CD8+ T-cell memory. Differentiation of CD8+ T-cell memory starts after naïve T-cell activation. According to the recently proposed model of progressive differentiation, the fate of T-cells depend on the duration of signaling and the presence or absence of cytokines (17). Thus, a single naïve cytotoxic T lymphocyte will differentiate gradually to different memory subsets: stem cell memory (T_{SCM}), central memory (T_{CM}), transitional memory (T_{TM}) cells, effector memory (T_{EM}), and terminally differentiated effector memory (T_{TE}). In consequence, brief antigen stimulation will generate T_{CM} cells and T_{TM} cells, the later being more differentiated than T_{CM} cells but not as fully differentiated as T_{EM} cells, in terms of phenotype. On the other hand, sustained stimulation together with presence of cytokines will generate T_{EM} and T_{TE} cells, which most probably include NK-like CD8+ T cells both circulating or tissue resident.

expression between T-cells and NK cells suggesting that NKR expression on T-cells is physiologically programed rather than a random event of the aging process (12). This may suggest that the NK-like CD8+ T-cells have a functional plasticity with respect to their "adaptation" that allows them to respond to different stimuli.

CMV and HIV infection strongly affect CD8+ T-cell differentiation and maturation, enhancing immunosenescence due to the accumulation of highly differentiated T_{EM} and T_{TE} cells (32, 33). The CD57 antigen has been traditionally used to characterize terminally differentiated "senescent" cells, as CD57+ T-cells exhibit a reduced proliferative capacity and altered functional properties (34). Of note, the expansion of $CD57^+CD8^+$ T-cells is a hallmark of latent CMV infection (35). The $CD57^+CD8^+$ T-cell subset is functionally heterogeneous, and includes highly cytotoxic T_{TE} cells that express intermediate levels of EOMES, as well as non-cytotoxic $EOMES^{hi}$ T_{TE} cells with high proliferative capacity (36). These above-referenced studies highlight the existence of functional heterogeneity among the CD8+ T-cell memory subsets. Regarding NK-like CD8+ T cells, high percentages of these cells also express the CD57 marker and likewise arise after CMV infection (10). It has been well established that NK-like CD8+ T-cells expand with age (30, 37–39) and some studies suggest that their frequency is increased in CMV-seropositive individuals (40–42). However, recent work performed in healthy young individuals indicates that NK-like CD8+ T-cell frequency is not affected by CMV latent infection. Thus, the authors propose that these cells accumulate with age in the CMV-seropositive individuals, rather than with CMV infection *per se* (10).

Moreover, NK-like CD8+ T-cells from Epstein–Barr virus (EBV)-associated tumor patients are quantitatively and functionally impaired and in a human-thymus-SCID chimera model, the EBV-induced human NK-like CD8+ T-cells synergize with NK-like CD4+ T-cells suppressing EBV-associated tumors upon induction of a Th1-bias (43). Additionally, in women with human papillomavirus (HPV)-associated cervical neoplasia, there are increased levels of $CD28^-$, T_{EM}, and $CD16^+CD56^+$ $CD8^+$ T-cells in peripheral blood, probably associated with the chronic infection with HPV (44). As we mentioned above, NK-like CD8+ T-cells possess a diverse TcR repertoire and there is evidence that these cells can function as antigen-specific suppressive cells that regulate the immune response through killing antigen-bearing dendritic cells (13). The class-I MHC-restricted T-cell-associated molecule (CRTAM) has been shown to be expressed only on activated class-I MHC-restricted T-cells, including NK-like CD8+ and conventional CD8+ T-cells. Of note, this molecule is a surface marker of activation associated with human viral infections and autoimmune diseases (45). These studies show that the NK-like CD8+ T-cells interact with other cells and that chronic stimulation determines their phenotype.

NK-LIKE CD8+ T-CELLS AND DISEASE

There is evidence in the literature of an immune suppressor role for the $CD8^+CD28^-$ T-cells (Ts) and the $CD3^+CD56^+$ T-cells. Patients with B-cell non-Hodgkin's lymphoma had significantly higher percentages of Ts cells and NKT-like cells than healthy people, suggesting that, in this type of lymphoma, these cell subsets may possibly have an immunosuppressive role (46). It has been suggested that tumor-induced dysfunction of CTL in patients with multiple myeloma may contribute to immune escape and causes clonal T-cell immunosenescence, but not exhaustion, as a predominant feature. These cells exhibited a senescent secretory effector phenotype: $KLRG\text{-}1^+/CD57^+/CD160^+/CD28^-$ (47) and may possibly be NK-like CD8+ T-cells with T_{EM} or T_{TE} phenotype. Furthermore, the use of *ex vivo*-expanded NK and NK-like T-cells has been reported seems to be safe and it could be an approach for further clinical evaluation in cancer patients (47).

Patients with Behcet's uveitis also showed an increased number of CD8+ T-cells and $CD8^+CD56^+$ (NKT-like) cells in the aqueous humor, indicating a possible role for these subsets in the immunopathogenesis of the disease (48). $CD56^+CD8^+$ NKT-cells express more IFN-gamma and KIR in patients with leishmaniasis compared with healthy subjects (49). Similarly, loss of CD28 was associated with an increased percentage of T and NK-like T-cells producing IFN-gamma or TNF-alpha in patients with chronic obstructive pulmonary diseases (44). Furthermore, targeting peripheral blood pro-inflammatory $CD28^-$ T-cells and NK-like CD8+ T-cells by inhibiting CD137 expression may possibly be of relevance to the treatment of bronchiolitis obliterans syndrome (50). In this regard, the percentage of $CD57^+CD8^+$ T-cells is the strongest immunologic predictor of future cutaneous squamous cell carcinoma and was correlated with increasing CD8+ T-cell differentiation (36). As mentioned above, a high percentage of $CD57^+CD8^+$ T cells are NK-like.

The human activating receptor NKG2D recognizes a diverse family of ligands (MICA, MICB, and ULBPs 1–6), leading to the activation of effector cells and triggering the lysis of target T-cells. Differential expression of NKG2D is regulated in the different T-cell subsets by epigenetic mechanisms (51). The NKG2D receptor–ligand system plays an important role in the immune

response to infections, tumors, transplanted grafts, and autoantigens. In lung cancer patients, NK-like CD8+ T-cells exhibit low expression of NKG2D, which correlates with the pathological stage (52). Thus, understanding the regulation of human NK-like CD8+ T-cells activation could be a strategy to manipulate T-cell-mediated responses including tumoral responses and infections.

Patients with Behcet's uveitis also showed an increased number of CD8+ T-cells and CD8+CD56+ (NKT-like) cells in the aqueous humor, indicating a possible role for these subsets in the immunopathogenesis of the disease (48). A skewed distribution and lower frequencies of circulating activated CD161+ NK-like CD8+ T-cells was observed in patients with common variable immunodeficiency disorders, suggesting a probable regulatory function of these cells (53). CD161 is expressed by several T-cell subsets, including CD8+, NK-like CD8+, CD4+, and TcR-gamma/delta cells and all CD161+ lymphocytes display a shared innate response to IL-12 + IL-18 in which CD161 can act as a costimulatory receptor (54). Additionally, IL-23 responsiveness is restricted to the CD161+ subset in CD45RO+CD8+ memory T-cells (55). Moreover, both the frequency and the absolute number of CD161+CD8+ T-cells are decreased in the peripheral blood of patients suffering from systemic lupus erythematosus (56). A skewed distribution and lower frequencies of circulating activated CD161+ NK-like CD8+ T-cells was observed in patients with common variable immunodeficiency disorders, suggesting a probable regulatory function of these cells (53).

Finally, evidence from murine research has shown that harnessing the immune adjuvant properties of NK-like CD8+ T-cells can be an effective strategy to generate immunological memory and anticancer immunity. This effect was associated with the IFN-gamma-dependent expansion of KLRG1+CD8+ effector T-cells (57). Another study in mice assessed the vaccine induction of CD8+ T-cell responses and protective immunity after coadministration of alpha-GalCer analog and TLR4 agonist. The results showed a robust CD8+ T-cell response to PyCS protein and WT-1 antigen and activation of memory-like effector NK-like CD8+ T-cells, with a CD44+CD62L− phenotype (14).

FUTURE RESEARCH CONSIDERATIONS

This review supports the concept that NK-like CD8+ T-cells are part of a cell subset associated with the acquisition of differential marker profiles, although there is little information regarding the functional properties of these cells in humans. Thus, there are several questions to clarify. First, are NK-like CD8+ T-cells CD3+/CD8+/CD56+ bright or dim? Second, are they TcR-alpha/beta+, TcR-gamma/delta+, or TcR-gamma/delta−? Third, do these cells contain variant or semi-invariant chains? Fourth, do they have CD8+/alpha-beta or CD8+/alpha-alpha? It would also be interesting to evaluate whether these subpopulations differ in their abilities to stimulate other immune cells and if they have diverse immunoregulatory functions including activation/suppression of diverse cells. Additionally, more information is needed with regards to how different environmental factors, such as autoimmunity, inflammation, viral antigen re-exposure, or persistent tumor antigens might allow the differentiation ("adaptation") of the memory NK-like CD8+ T-cells. Finally, it is important to be cognizant of the different NK/T-cell-like cell populations and exclude the NK-like CD8+ T-cells when analyzing the immune responses mediated by conventional CD8+ T-cells and vice versa.

Nutrient/metabolic regulators can influence NK-like CD8+ T-cell differentiation, which could be analyzed through nutrigenomics contributing to the knowledge regarding the differentiation of this subset. As the balance between activating/inhibitory receptors controls NK-like CD8+ T-cells immune responses, it should also be considered that the expression of these receptors could depend on the cell differentiation state, the age, and/or diseases of the individual.

In conclusion, a thorough functional and phenotypic characterization of human NK-like CD8+ T-cells will be fundamental in order to provide mechanistic insight into the functional adaptation of these cells to aging, autoimmunity, inflammation, viral, and tumor antigens and toward their exploitation in potential therapeutic applications.

AUTHOR CONTRIBUTIONS

M-PL conceived and participated in the design and coordination of the manuscript. AP and RS provided helpful discussions and edited the manuscript. All authors wrote, read, and approved the final manuscript.

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REVIEW
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Molecular and Cellular Characterization of Human CD8 T Suppressor Cells

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Bidirectional interactions between dendritic cells and Ag-experienced T cells initiate either a tolerogenic or immunogenic pathway. The outcome of these interactions is of crucial importance in malignancy, transplantation, and autoimmune diseases. Blockade of costimulation results in the induction of T helper cell anergy and subsequent differentiation of antigen-specific CD8$^+$ T suppressor/regulatory cells (Ts). Ts, primed in the presence of inhibitory signals, exert their inhibitory function in an antigen-specific manner, a feature with tremendous clinical potential. In transplantation or autoimmunity, antigen-specific Ts can enforce tolerance to auto- or allo-antigens, while otherwise leaving the immune response to pathogens uninhibited. Alternatively, blockade of inhibitory receptors results in the generation of cytolytic CD8$^+$ T cells, which is vital toward defense against tumors and viral diseases. Because CD8$^+$ T cells are MHC Class I restricted, they are able to recognize HLA-bound antigenic peptides presented not only by APC but also on parenchymal cells, thus eliciting or suppressing auto- or allo-immune reactions.

Keywords: CD8$^+$ T suppressor cells, ILT3, co-stimulation blockade, transplantation, autoimmune disease, gene profile of CD8$^+$ Ts

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INTRODUCTION

Over the last decade, the prevailing dogma has been that self-tolerance is mediated through dominant suppression of autoimmune responses by regulatory CD4$^+$CD25$^+$FoxP3$^+$ T cells (CD4$^+$ Treg). Naturally occurring Tregs specifically express the transcription factor FOXP3 (forkhead box P3) (1). Natural CD4$^+$CD25$^+$ Treg constitute 5–10% of peripheral CD4$^+$ T cells in normal mice and <5% in humans. Their essential role in tolerance was shown by experiments in which the depletion of natural Tregs from the thymus of newborn rodents resulted in enhanced immune responses to conventional bacteria from the intestine. This provoked inflammatory bowel disease (IBD) and the development of autoimmune diseases. In contrast, expansion of Tregs suppressed allergy, organ allograft rejection, graft-versus-host disease after bone marrow transplantation, and various autoimmune diseases (1–5).

The revival of CD8$^+$ suppressor cells (CD8$^+$ Ts) after decades of deliberate omission has been well described in some review articles (6–8). The function of CD8$^+$ Ts was first documented in the early 80s by Gershon et al. (9). With the advent of molecular immunology, the existence of the murine I-J locus, presumed to encode Ts function, could not be confirmed. For fear of rejection and denial of grant support, the word "suppressor" was arbitrarily replaced with that of "regulatory" T cells, even

though the sole function of regulators was to suppress immune function. For this reason, the reader of the suppressor literature would be well advised to search the listing of papers referring to either $CD8^+$ Treg or T suppressor cells ($CD8^+$ Ts).

Both $CD8^+$ and $CD4^+$ Tregs showed similar expression levels of FOXP3 and CTLA-4, which represent their most characteristic markers. On the other hand, the biggest difference between $CD4^+$ and $CD8^+$ Tregs resides in the expression of CD28 (10). $CD4^+$ Tregs express a higher level of CD28, which is required for their interaction with B7 molecules. B7 molecules regulate thymic development and peripheral tolerance (11). For $CD8^+$ T cells, the expression of CD28 is partially dispensable due to their reduced production of IL-2 (12–14).

NATURAL AND NON-ANTIGEN-SPECIFIC $CD8^+$ Treg

Similar to natural $CD4^+$ Treg, $CD8^+$ thymus-derived natural Tregs have also been described. Characteristically, these cells have a $CD28^-$ phenotype in both mice and human (15). However, after TCR triggering, both $CD4^+$ and $CD8^+$ natural Treg inhibit the immune response in an antigen non-specific and MHC non-restricted manner *via* direct interaction between Treg and activated T cells. Naturally occurring $CD8^+$ Treg were reported to have a $CD8^+CD25^+CTLA\text{-}4^+GITR^+FoxP3^+$ phenotype and suppress in a CTLA-4- and TGF-β1-dependent manner (16).

The Qa-1-restricted CD8 alpha, alpha$^+$ (TCR alpha beta$^+$), population is the best characterized population of $CD8^+$ natural Treg in mice. The Qa-1 molecule (homolog of HLA-E in human) presents peptides derived from the non-hypervariable domain of the TCR. These Vbeta-specific $CD8^+$ Tregs interact and inhibit the activation of $CD4^+$ T cells with similar Vbeta regardless of their specificity (17–20).

Study of the miRNA profile of human $CD8^+CD25^+$ natural Treg revealed 10 differentially expressed miRNAs (miR-214, -205, -509 overexpressed and miR-9, -24, -31, -155, -335, -210, and -449 under expressed), which seem to display specific regulation of FOXP3, CTLA-4, and GARP gene expression (21).

Peripheral $CD8^+$ $CD28^-$ $Foxp3^-$ $CD56^-$ non-antigen-specific Ts were reported to be easily generated and expanded by culturing $CD8^+CD28^-$ T cells in a cocktail of cytokines containing IL-2, IL-10, and GM-CSF. They were expanded without antigenic stimulation and seemed to inhibit antigen recognition, T cell proliferation, and cytotoxicity *via* IL-10 secretion (22, 23). It has been suggested that such Ts can be extracted from patients during disease remission and reinfused during disease exacerbation (24).

ADAPTIVE ANTIGEN-SPECIFIC $CD8^+$ Treg

Adaptive $CD8^+$ Ts originate from the post-thymic T cell pool and are induced by a variety of *in vivo* and *in vitro* antigenic stimuli. Antigen-specific Treg are required for efficient suppression of T cell immune responses against MHC-bound peptides derived from auto- or allo-antigens. The best characterized Treg in this category include human $CD8^+CD28^-$, MHC class I-restricted, T suppressor, and $CD4^+CD25^+CD45RO^+$, MHC class II-restricted, Treg cells (10). Our previous studies have demonstrated that MHC allo-restricted $CD8^+CD28^-$ Ts can be generated *in vitro* by multiple rounds of T cell stimulation in the presence of allogenic APC. Evidence has been provided that Ts develop *in vivo* from rejection-free organ allograft recipients. Antigen-specific $CD8^+CD28^-$ Ts exert their function by conditioning APC to become tolerogenic. Our studies on the mechanism of $CD8^+CD28^-$ Ts-mediated suppression revealed that they act *via* an APC bridge, inducing the upregulation of immunoglobulin-like transcript (ILT) inhibitory receptors on professional (dendritic cell and monocytes) as well as on non-professional [endothelial cells (EC)] APC (25–29).

$CD8^+$ Ts AND ILT3

The induction of tolerogenic dendritic cells (DCs) was first established in 1998 by our group (26). We showed that human $CD8^+CD28^-$ Ts cells generated by multiple rounds of *in vitro* allo-stimulation interact with APC, inducing the downregulation of co-stimulatory molecules and thereby reducing their capacity to trigger $CD4^+$ T helper (T_h) cell activation (27). In the absence of T_h cell help, $CD8^+$ T cells from the same culture acquire suppressor activity. Similarly, multiple stimulations of human T cells with xenogeneic APC or with peptide-pulsed autologous APC resulted in the generation of antigen-specific $CD8^+CD28^-$ Ts cells (28, 29). These $CD8^+$ Ts cells, derived from an oligoclonal population, are MHC class I restricted and express same levels of FOXP3, GITR, CTLA-4, CD25, OX40, CD103, CD62L, 4-1BB, and TNFRII as seen in $CD4^+CD25^+$ natural T regulatory (Treg) cells (10, 30).

$CD8^+CD28^-$ Ts can be distinguished from $CD8^+CD28^-$ CTL cells from the same multiple allo-stimulated T cell line (TCL) by the higher expression of some genes from the killer cell inhibitory receptor (KIR) family, such as KIR3DL1, KIR3DL2, and KIR2DL3 and by their gene profile (10).

Upon restimulation with priming APC, $CD8^+$ Ts do not produce IFN-γ, IL-10, TGF-β, or other cytokines. Instead, $CD8^+CD28^-$ Ts inhibit CD40-mediated upregulation of co-stimulatory molecules, such as CD80 and CD86 on priming APC, which become tolerogenic, upregulating the expression of the inhibitory receptors ILT3 (also called LILRB4, CD85K, or LIR5) and ILT4 (also known as LIR-2, LILRB2, or CD85d). Consequently, APC are rendered unable to induce and sustain the full program of $CD4^+$ T_h cell activation and maturation, due at least in part to inhibition of Nuclear Factor-κB (NF-κB) activation and subsequent transcription of co-stimulatory molecules (10, 26–29, 31–34).

Tolerogenic APC can be also generated by exposure of DC to IL-10, IFN-α, or IFN-β, which induce upregulation of ILT3 and ILT4 (14–17, 31–34).

The crucial role of ILT3 and ILT4 was revealed in experiments in which the myelomonocytic cell line KG1 was transfected with ILT3 or ILT4 and used for T cell allo-stimulation. Wild KG1 cells induced strong MLC responses, while ILT3- or ILT4-transfected KG1 cells were non-stimulatory. $CD8^+CD28^-$ T cells from the same cultures inhibited autologous T cell responses to wild KG1 stimulating cells, displaying suppressor function. $CD4^+$

T_h reactivity to KG1-ILT3 or KG1-ILT4 transfectants could be restored by adding to the cultures either anti-ILT3 or ILT4 mAb, respectively, or IL-2. These results indicated that ILT3- or ILT4-expressing APC induce T cell anergy and elicit the differentiation of $CD8^+CD28^-$ Ts (26).

Allogeneic CD40L-activated pDC (expressing high levels of ILT3 and ILT4) promote the differentiation of naïve CD8 T cells into $CD8^+$ Ts. These $CD8^+$ Ts inhibit T cell proliferation *via* secretion of IL-10 (35).

While overexpression of ILT3 was shown to be a marker of tolerogenicity, knock down of ILT3 augmented the immunogenic capacity of activated DC, significantly increasing their capacity to migrate, produce inflammatory cytokines, and activate IFN-γ- and IL-17-secreting T effector cells (36).

ILT3 and ILT4 belong to a family of Ig-like inhibitory receptors that are structurally and functionally related to KIRs. Some ILT family members, including ILT2, ILT3, and ILT4, have long cytoplasmic tails containing ITIM. These receptors mediate inhibition of cell activation by recruiting the tyrosine phosphatase SHP-1 (37–40). Coligation of ILT3 and ILT4 in monocytes inhibits Ca^{2+} mobilization and tyrosine phosphorylation, which is triggered by Ab ligation of FcRII (CD32), HLA-DR, and FcγRI (CD64). The ligand for ILT3 has not been described so far. ILT4 was shown to bind to the α3 domain of HLA class I (HLA-A, HLA-B, HLA-C, and HLA-G), competing with CD8 for MHC class I binding (41, 42). As a result, recombinant soluble ILT4 restores, rather than inhibits, Treg proliferation (43).

Besides the negative signaling that ILT3 transmits endogenously upon ligation, ILT3's extracellular Ig-like domains are also endowed with inhibitory function. This was demonstrated in experiments for which we first engineered the myelomonocytic KG1 cell line (KG1-Delta) to overexpress a signaling-defective ILT3 deletion mutant which lacked the cytoplasmic tail containing ITIM. $CD4^+$ T-cell responses in primary and secondary MLC were greatly deficient upon stimulation with these cells, which elicited instead the generation of $CD8^+CD28^-$ Ts cells. This result suggested that the extracellular domain of ILT3 by itself carries out a tolerogenic function which is independent of the inhibitory intracellular signaling (43). Based on these findings, we engineered a recombinant ILT3.Fc protein, which lacked both the trans-membrane and intracellular domain. When ILT3.Fc was added to T cells at the time of MLC priming, it suppressed $CD4^+$ T_h cell proliferation and elicited the differentiation of allospecific $CD8^+$ Ts *in vitro* as well as *in vivo*. Hence, both membrane and soluble ILT3 induce $CD4^+$ T helper anergy, triggering the generation of $CD8^+$ Ts cells (41, 44, 45). This indicates that ILT3 is an essential immune checkpoint or master switch which regulates the outcome of the immune response.

Soluble ILT3, engineered as an ILT3.Fc fusion protein, was shown to induce tolerance to allogeneic human pancreatic islet transplants in humanized NOD/SCID mice (hu-NOD/SCID) (44) and to reverse progression of rejection after its onset (34). ILT3.Fc inhibited both the cellular and humoral arm of rejection, as shown by the inhibition of T_h1 and T_h2 proliferation and cytokine production, CTL generation, and synthesis of anti-HLA and xenospecific antibodies by B cells from tolerant animals (34, 44, 46).

GENE PROFILE OF ILT3.Fc-INDUCED $CD8^+$ Ts

ILT3.Fc dramatically changes the landscape of the gene expression profile in $CD8^+$ Ts cells. Numerous genes in the WNT receptor pathway were significantly upregulated, indicating its important role in the generation of $CD8^+$ Ts cells. This data support the concept that activation of the WNT pathway inhibits CD8 T-cell proliferation and cytotoxic effector cell differentiation. The expression of TGF-β and TGFBR2 was also significantly increased, consistent with the well-characterized cross talk between TGF-β and WNT pathway (47, 48).

ILT3.Fc extensively downregulated the expression of cyclins and cyclin kinases while upregulating cyclin-dependent kinase inhibitors. Considering the fact that cyclins and cyclin kinase together with their specific inhibitors are the most important positive and negative regulators in the cell cycle, it is reasonable to assume that ILT3.Fc induces cell cycle arrest, inhibiting T cell proliferation (47, 49).

On the gene transcriptional level, ILT3.Fc promotes the expression of transcriptional repressors which block the synthesis of cytokines and other factors necessary for T cell proliferation and differentiation. The zinc finger transcriptional repressor BCL6 is one of the genes whose elevated expression is important for the differentiation of ILT3.Fc-induced Ts. We found that transfection of BCL6 in allo-activated $CD8^+$ T cells converted them into suppressors, whereas silencing of BCL6 in unprimed T cells prevented their differentiation into Ts when allo-stimulated in the presence of ILT3.Fc. BCL6-transfected Ts share highly similar characteristics with ILT3.Fc-induced Ts both *in vitro* and *in vivo*. The *in vivo* evidence is based on the finding that BCL6 was overexpressed in human $CD8^+$ T cells from humanized mice rendered tolerant to pancreatic islet transplants by treatment with ILT3.Fc (34). ILT3.Fc-induced repression of granzyme B, IFN-γ, IL-5, and enhancement of CXCR4 occurred in conjunction with the upregulation of BCL6 expression in $CD8^+$ Ts cells. Hence, ILT3.Fc may arbitrate T cell lineage fate through BCL6-mediated repression of T_h1, T_h2, T_h17, and CTL and induction of Ts differentiation (34).

MiRNA represents a group of novel regulatory molecules which modulate gene function at the posttranscriptional level. Studies on the miRNA expression profile in ILT3.Fc-induced $CD8^+$ Ts indicate that they also play a role in the generation of $CD8^+$ Ts cells. ILT3.Fc inhibited the expression of miR-21, miR-30b, and miR-155. Those miRNAs target the 3′-untranslated region of DUSP10, BCL6, and SOCS1, genes whose transcription was highly increased in ILT3.Fc-induced Ts.

Primed $CD8^+$ T cells transfected with miR-21 and 30b, miR-21 and 155, or miR-21, 30b, and 155 inhibitors displayed suppressor activity when added to autologous CD3-triggered $CD4^+$ T cells. Luciferase reporter assays of miR-21 and miR-155 indicated that their transcription is highly AP1 dependent, consistent with the finding that for the AP1 subunits, FOSB, and c-FOS, translocation to the nucleus is inhibited by ILT3.Fc. In summary, ILT3.Fc inhibits T cell activation and induces the generation of Ts by targeting multiple inflammatory miRNA pathways (50). Recent studies on human natural $CD8^+CD25^+FOXP3^+CTLA\text{-}4^+$ Treg

cells revealed similar miRNA signatures. The data indicate that miRNAs, including miR-9, -24, -155, and -335, play an important role in the induction of $CD8^+$ Treg by modulating Treg-associated genes (21).

Studies of exosomes from MLC supernatants revealed the presence of inflammatory microRNA, including miR-146a, miR-155, miR-21, miR-30b, miR-365, and Let-7a. These miRNAs were inhibited when ILT3.Fc was added to the culture, they were produced exclusively by $CD4^+$ T cells, being absent from CD4-depleated cultures. Furthermore, upon treatment with exosomes containing inflammatory microRNA, ILT3. Fc-induced $CD8^+$ Ts lost their suppressive activity at low Ts/T effector cell ratio (51).

MiRNAs contained by exosomes released from allo-activated T cells enhanced T helper activity even in the presence of limiting amounts of allospecific T suppressor cells. This suggests that increased amounts of microRNA in recipients' sera may serve as markers of active immune responses against the graft, even in the presence of regulatory T cells. Furthermore, such exosomes may be of use in eradicating tumors in patients developing lymphoid malignancies secondary to immunosuppression and viral (EBV, CMV, Hepatitis B and C) infections (51).

Comparison of ILT3.Fc-induced $CD8^+$ Ts with $CD8^+CD28^-$ Ts induced in MLC by chronic allogenic stimulation demonstrated that the characteristic signatures of $CD8^+$ T suppressor cells generated by either of these methods are the same, consisting of upregulation of the BCL6 transcriptional repressor and downregulation of inflammatory microRNAs, miR-21, miR-30b, miR-146a, and miR-155. In conclusion, microRNAs, which are increased under inflammatory conditions in activated $CD4^+$ and $CD8^+$ T cells with helper or cytotoxic function, show low levels of expression in $CD8^+$ T cells that have acquired antigen-specific suppressor activity (52).

$CD8^+$ Ts IN TRANSPLANTATION

The possible role of ILT3 and ILT4 molecules in maintenance of quiescence in transplant patients is of obvious interest. We found that T cells from heart and liver transplant patients in quiescence, but not from recipients with a history of rejection episodes, induced the upregulation of ILT3 and ILT4 and downregulation of CD80 and CD86 in cryopreserved APC from the donor. As a surrogate for donor APC, DC matched to the donor for at least one HLA class I and one HLA Class II (DR) can be used for flow cytometry and functional assays.

Monitoring of kidney allograft recipients who have been chronically exposed to rapamycin showed increased numbers of DC with the $ILT3^+ILT4^+$ tolerogenic phenotype and of T cells with the $CD8^+CD28^-$ suppressor phenotype suggesting that mTOR inhibition promotes a novel immunoregulatory pathway (53).

However, since donor DC migrate out of the graft early following transplantation, it was still unclear how quiescence was maintained by some, but not all organ allograft recipients. The most likely explanation seems to be that graft EC, which are non-professional APC that express all donor HLA allo-antigens, become tolerogenic. To explore the possibility that EC are targeted by recipient $CD8^+CD28^-$ Ts, we transfected umbilical cord EC (matched to the donor for at least one HLA class I antigen) with luciferase ILT3 or ILT4 reporter gene and performed luciferase transcription assay in the presence of recipient $CD8^+CD28^-$ T cells. These experiments demonstrated that $CD8^+CD28^-FoxP3^+$ T cells from the circulation of rejection-free heart transplant patients triggered the expression of ILT3 and ILT4 in EC-sharing class I HLA antigens with the graft. $CD8^+$ T cells from patients with recurrent episodes of acute or with chronic heart allograft rejection did not display ILT3-inducing capacity (54, 55). Using cell fractionation and sequencing studies, we further showed that ILT3 precursor RNA are expressed and retained in the nuclei of resting EC. Ts interaction with EC or exposure of EC to IL-10 and IFN-α triggers processing of ILT3 pre-mRNA. Western blot analysis showed that the expression of the mature ILT3 transcript is accompanied by production of ILT3 protein (56). Studies from other laboratories further confirmed our finding that IL-10 also inhibits endothelium-dependent T cell co-stimulation by upregulating ILT3 and ILT4 in human vascular EC (57).

The tolerogenic role of EC, which express inhibitory molecules, was further explored in a Lewis to ACI rat heart transplantation model. After three injections of UV-irradiated blood from Lewis donors, about 50% of the ACI recipients became tolerant to donor strain heart transplants. Tolerance could be transferred to secondary ACI recipients by $CD8^+$ but not by $CD4^+$ T cells. Furthermore, the graft of these secondary recipients was tolerated indefinitely even when transplanted to tertiary, non-conditioned ACI recipients. The $CD8^+$ T cells used for adoptive transfer of tolerance were $FoxP3^+$. They induced the expression of PIR-B, a rat ortholog of ILT4, not only in donor APC, but also in the EC lining the aorta of the transplanted heart. Hence, this phenomenon of "graft adaptation" was mediated by the induction of inhibitory receptors in graft EC by MHC Class I allo-restricted $CD8^+$ suppressor cells (58).

Recently, it was shown that kidney–pancreas transplantation in a type I diabetic patient was characterized by an increased presence of $CD8^+CD28^-$ Treg in the pancreas and elevated levels of ILT3 expression on APC in a donor-specific manner (59).

Our conclusion that generation of allospecific $CD8^+CD28^-$ Ts may require the induction of anergy in $CD4^+$ T helper cells (T_h), e.g., leaving the primed $CD8^+$ T cells "helpless," is supported by other groups. These investigators used the same MLC stimulation model, but "allo-anergized" T_h by adding to the MLC a CTLA-4 immunoglobulin fusion molecule (Belatacept) which blocks the CD28-B7 co-stimulation pathway. They further confirmed our finding that repeated rounds of allo-stimulation results in relative and absolute expansion of $CD8^+Foxp3^+$ Ts. Finally, they found that allo-anergized $CD8^+CD28^-$ Ts of donor origin are expanded in recipients of allogeneic hematopoietic stem cell transplantation (60). This finding is reminiscent of our and other authors' previous observation that $CD8^+CD28^-$ Ts are present in the circulation of transplant recipients in quiescence (26, 61–63).

Human allo-antigen-specific $CD8^+$ Tregs were generated in a large scale from antigenically naïve precursors by *in vitro* stimulation with allogeneic CD40 activated B cells. These cells inhibited GVHD in a humanized mice model, suppressing allo-reactive

T cell proliferation and cytokine production by a CTLA-4-dependent mechanism (64). In other studies, $CD8^+CD28^-$Tregs were generated in MLC by coculture with mesenchymal stem cells, a method of potential use given the resistance of $CD28^-$ T cells to treatment with Belatacept, an agent which blocks CD28/B7 co-stimulation, preventing alloreactivity in kidney transplant recipients (65).

In recent studies, $CD8^+CD28^-$ Ts cells were generated by multiple MLC simulation and expanded by adding common gamma chain cytokines IL-2, IL-7, and IL-15 to the cultures. The expanded population exhibited increased expression of CTLA-4, FOXP3, and CD25, while the expression of CD56, CD57, CD127, and perforin was downregulated (66). Consistent with our own studies, suppression of CD4 T_h by $CD8^+CD28^-$ Ts was HLA class I allo-restricted and cytokine independent. However, the claim that after expansion these cells keep their suppressor function without killing the stimulating APC, as demonstrated in CFSE cytotoxic assays, could not be confirmed in our own studies for which the traditional Cr-51 release from target cells was used. We found that when these cytokines were used for expansion, the proliferating population consisted of $CD8^+$ CTL. It is apparent that rather than being in a terminal stage of differentiation, $CD8^+CD28^-$ Tcells can be expanded indefinitely given the appropriate mixture of cytokines (67). A similar phenomenon has been described for $CD4^+$. Zhou et al. have shown that $CD4^+$ Tregs constitute an unstable T cell subset that can be reprogramed into pathogenic effector cytokine-producing T cells. Such $CD4^+$ T cells down-regulate their FOXP3 expression, losing suppressive capacity and acquiring an activated-memory phenotype (68). These findings reflect the plasticity of both $CD8^+$ and $CD4^+$ Treg cells, which can revert their function from suppressors to effector cells. A comprehensive review of $CD8^+$ Treg in animal transplantation studies can be found in Guillonneau et al., which also emphasizes the identification of PD-1 as a marker of $CD8^+$ Ts in rodents (69).

Recently, a new subset of $CD8^+CD122^+PD1^+$ Tregs, which produce IL-10, IFN-γ, and TGF-β and suppress $CD4^+$ T cell activation, has been described in mice. Its counterpart in human is still unknown (70). This $CD8^+cd122^+$ Treg subset was claimed to more potently suppress allograft rejection compared to their $CD4^+CD25^+$ Tregs (71). Therefore, it appears that $CD8^+CD28^-$, $CD8^+Qa\text{-}1^+$, $CD8^+CD103^+$, and $CD8^+CD122^+PD\text{-}1^+$ Treg subsets may share the capacity of maintaining homeostasis with an equally heterogeneous population of $CD4^+$ Tregs.

$CD8^+$ Ts CELLS IN AUTOIMMUNE DISEASES

$CD8^+$ T cells can oppose or promote autoimmune disease through activities as suppressor or as cytotoxic effectors (72). Data have been presented that $CD8^+CD28^-CD56^-$ T cells have suppressive activity in rheumatoid arthritis (RA), preventing the activation of naïve $CD4^+$ T cells and inhibiting their effector function *in vivo*. When transferred into NOD-SCID chimeras engrafted with human synovial tissue, they suppressed the inflammatory activity in the synovial lesions and inhibited cytokine production (73). Rheumatoid synovitis could also be treated in such chimeras by infusing autologous $CD8^+CD16^+$ T cells, which inhibits the production of IL-1β, IFN-γ, TNF-α, and other inflammatory cytokines. Treatment with IL-16 mimicked the effect of the adoptive transfer of Ts and anti-CD16 antibodies abrogated the suppressor effect (74). Similarly, there is evidence that $CD8^+$ Ts generated from SLE patients during remission had suppressor activity, while $CD8^+$ obtained during exacerbation of the disease had no such activity (75).

Glatiramer acetate (GA) introduced in the therapy of multiple sclerosis has been shown to induce $CD8^+$ Ts, which seem to recognize GA on the cell surface and directly kill $CD4^+$ T cells in a HLA-E-dependent manner (76). Ulcerative colitis and Crohn's disease are other examples of pathological processes in which $CD8^+$ Ts may inhibit proliferation of $CD4^+$ T cell through a TGF-β-dependent mechanism (77). Intestinal epithelial cells may activate $CD8^+$ Ts cells, which downregulate IBD, suppressing IgG production (78). In JDM, a one-course administration of humanized anti-CD3 mAb was claimed to induce the generation of $CD8^+$ $CD25^+CTLA\text{-}4^+FoxP3^+$ Ts cell, which were able to inhibit the stimulation of $CD4^+$ T cells in *in vitro* coculture system (79, 80). Accumulating evidence indicates that co-inhibitory molecules, such as CTLA-4, PD1, and BTLA, are negative regulators of immune responses since deficiencies or mutations result in the development of autoimmune diseases. The administration of decoy co-inhibitory receptors, such as CTLA-4.Ig or agonistic antibodies, can suppress the response of self-reactive T cells in autoimmune diseases.

Abatacept, a fusion protein composed of the Fc fragment of human IgG1 linked to the extracellular domain of CTLA-4, has shown efficacy in a broad spectrum of RA patients from early stage to refractory diseases that are resistant to TNF blockers (81–84).

In addition, Abatacept showed efficacy in patients with juvenile idiopathic arthritis, psoriasis, and SLE. CTLA-4 competes with CD28 on the membrane of activated T cells for binding to B7 molecules (CD80 and CD86) on APC, delivering negative signals which inhibit or terminate T cell responses.

PD1, another co-inhibitory receptor from the CD28 family, which is expressed on activated T cells (as well as on B cells and monocytes), binds to two ligands of the B7 family, PD-L1 and PD-L2. The ligation of PD-1 with these ligands inhibits $CD4^+$ and $CD8^+$ T cell proliferation by arresting the cell cycle (85–87). However, in human, PD1 ligation is important to inhibition of cell proliferation and death by apoptosis, rather than to the generation and function of $CD8^+$ Ts.

BTLA-HVEM pathway is another inhibitory pathway for lymphocyte activation [reviewed in Ref. (78)]. BTLA4, a member of the TNF family, is expressed on $CD4^+$ and $CD8^+$ T cells, NK and NKT cells, as well as on B cells, DC, and macrophages. Its ligand HVEM is also widely expressed on hematopoietic cells, including T cells and APC. Ligation of BTLA induces its tyrosine phosphorylation and SHP-1/SHP-2 association, inhibiting T cell proliferation and IL-2 production. Deficiency of BTLA-HVEM interaction has been shown to be involved in autoimmune diseases, though no clinical trial is yet in progress.

Although current methods of immunotherapy in cancer are largely based on the use of the immune checkpoint inhibitors anti-CTLA-4 and anti-PD-1 antibodies, the major obstacle

resides in opportunistic autoimmune disorders and associated morbidity resulting from altered immune regulation (88, 89).

Other lymphocyte subsets believed to have suppressor function in autoimmune diseases and transplantation include $CD3^+CD4^-CD8^-$ (double negative Treg), $CD4^+$, Valpha14 negative (NKTreg), and gamma/delta Treg cells. It was postulated that there are four modes of Treg function: (1) secretion of inhibitory cytokines such as IL-10 and TGF-β; (2) granzyme-perforin-induced apoptosis of effector T lymphocytes; (3) induction of apoptosis by deprivation of cytokines; and (4) inhibition of DC function (8, 90).

Specific recognition of the MHC Class Ib Qa-1bound peptides expressed on activated CD4 T cells by regulatory, cytolytic $CD8^+$ T cells was postulated to prevent autoimmunity in mice (91). A similar function has been attributed to human neuroantigen-specific $CD8^+$ Treg, which recognize HLA-E-bound peptides and are present in the circulation of patients with multiple sclerosis (92).

A distinct subset of human $CD8^+CD25^+FoxP3^+$ Treg seems to be characterized by the expression of the lymphocyte activation gene-3 (LAG-3). The suppressive activity of this subset has been attributed to the secretion of the CC chemokine ligand 4 (CCL4), which interferes with TCR signaling, inhibiting T cell activation. These Tregs can be expanded only from T cells primed *in vivo* to a specific antigen by repeated or chronic stimulation *in vitro* or *in vivo*, respectively. This indicates that they are adaptive Treg (90).

It has been shown that autologous hematopoietic stem cell transplantation in refractory SLE can induce immunological tolerance to auto-epitopes from nucleosomes by restoring the $CD8^+FoxP3^+$ TGF-β producing pool of suppressors. These Ts maintained high expression levels of latency-associated peptide (LAP), CD103, PD-L1, and CTLA-4 following transplantation and completely inhibited autoimmunity (93).

Collectively these data indicate the importance of $CD8^+$ Treg in suppressing autoimmune responses and transplant rejection.

$CD8^+$ REGULATORY T CELLS IN PERSISTENT VIRAL INFECTION

The control of virus-specific immune responses may be a mechanism that permits virus persistence. On the other hand, it may also protect the patient from overwhelming T cell reactivity and destruction of infected tissues (94).

In patients with HIV, it was shown that stimulation of patients' PBMC with HIV-specific antigens induced TGF-β-producing $CD8^+$ Treg. These $CD8^+$ Treg suppressed the IFN-γ production of HIV-specific and vaccinia virus-specific $CD8^+$ T cells, displaying both a specific and non-specific activity. IL-10-producing $CD8^+$ Treg were also expanded from the peripheral blood of HIV-infected individuals and their frequency seems to be associated with impairment of $CD8^+$ effector-cytolytic function (95–97).

Distinct populations of $CD8^+$ Ts were also shown to be present in the blood and liver of patients with chronic HCV infection. Some investigators reported the expansion of $CD25^-FoxP3^-CD8^+$ Treg that inhibited IFN-γ production of HCV-specific $CD4^+$ and $CD8^+$ T cells *via* TGF-β production. A population of HCV-specific $FoxP3^+CD8^+$ Treg was also expanded by stimulation with HCV peptides of PBMC from patients with chronic HCV infection. These cells inhibited in an antigen non-specific manner, T cell proliferation *via* direct T cell–T cell interaction (98, 99). Similarly, expansion of virus-specific $CD8^+BTLA^+$ T cells in the liver was observed in patients with chronic HBV infection. These infiltrating regulatory T cells were antigen specific and suppressed T cell responses *via* IL10 secretion (100).

Intra-hepatic IL-10-secreting $CD8^+$ Treg were described to be present in patients with chronic infection and to suppress IFN-production of effector $CD8^+$ T cells primed to the same HCV peptide. Blockade of IL-10 restored effector activity (101).

Herpes viruses, such as EBV, CMV, or HSV, which infect many people worldwide, establish persistent latent infections which, upon reactivation in immunological deficient individuals, are responsible for life threatening episodes of infection. EBV-specific $CD8^+FoxP3^+$ Treg cells produce IFN-γ and IL-10 but not TGF-β and suppress $CD4^+$ T cell proliferation in a cell–cell contact manner. Similar mechanisms seem to occur in CMV infection (102, 103).

Evidence has been provided that dermal $CD14^+$ DCs, which express ILT2 and ILT4, prime a fraction of naïve $CD8^+$ T cells that produce type 2 cytokines (IL-4 and IL-5) as opposed to Langerhans cells, which have no ILTs and are highly efficient in priming $CD8^+$ CTL. The ILT molecules on dermal DC polarized the T cell response toward type2 cytokine producers, as blocking of these receptors enhanced the generation of CTL (104).

Accumulating evidence revealed that viruses have evolved strategies to evade the immune surveillance by inducing specific Tregs. Novel strategy show promising antiviral effects by deletion or inactivation of viral-induced Tregs cells (105).

CONCLUSION

In light of the multiple pathways which may lead to the activation, generation, and expansion of T cells with antigen-specific function, it is obvious that therapy based on enhancement or blockade of immune checkpoints used by T cells for interacting with other cells holds promising results. Recombinant ILT3.Fc, CTLA-4. Ig, PD-L1.Ig, or humanized monoclonal antibodies which block effector–ligand interaction are only examples of the numerous agents that may have beneficial effects. However, the success of novel therapies aimed at suppression of autoimmune diseases depends on progress in certain areas:

1. It is apparent that inhibition of immune responses to unidentified autoantigens, deriving from a variety of tissues, calls for a better understanding of signaling pathways activated by ligation of different co-inhibitory checkpoints. This may allow for the design of combination therapy in which agents which act in synergy can be used to inhibit the activation and maturation of effector T helper and cytotoxic cells.
2. Studies on mechanisms' underlying memory of both effectors and suppressors of T cell immune responses to defined antigens may permit the design of clinical protocols which maintain quiescence in patients in remission.
3. Identification of markers that characterize different stages of T cell conversion from one effector function to another,

as exemplified by $CD8^+$ suppressor and cytotoxic T cells, as well as of the mechanism of such a transition, is required for patients' monitoring and better timing of therapy.

4. Progress in understanding the way in which cells communicate with each other to perceive endogenous and exogenous signals may open new horizons to immunotherapy of autoimmune diseases and of cancer.

AUTHOR CONTRIBUTIONS

ZX drafted the paper, organized its content, and collected and summarized the data. SH participated in the editorial process, data collection, and analysis. C-CC, Q-YZ, E-RV, and GV performed the critical revision of this article. NS-F is the PI of the study and made the ultimate decision on the manuscript.

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Divide, Conquer, and Sense: $CD8^+CD28^-$ T Cells in Perspective

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Understanding the rationale for the generation of a pool of highly differentiated effector memory $CD8^+$ T cells displaying a weakened capacity to scrutinize for peptides complexed with major histocompatibility class I molecules *via* their T cell receptor, lacking the "signal 2" CD28 receptor, and yet expressing a highly diverse array of innate receptors, from natural killer receptors, interleukin receptors, and damage-associated molecular pattern receptors, among others, is one of the most challenging issues in contemporary human immunology. The prevalence of these differentiated $CD8^+$ T cells, also known as $CD8^+CD28^-$, $CD8^+KIR^+$, NK-like $CD8^+$ T cells, or innate $CD8^+$ T cells, in non-lymphoid organs and tissues, in peripheral blood of healthy elderly, namely centenarians, but also in stressful and chronic inflammatory conditions suggests that they are not merely end-of-the-line dysfunctional cells. These experienced $CD8^+$ T cells are highly diverse and capable of sensing a variety of TCR-independent signals, which enables them to respond and fine-tune tissue homeostasis.

Keywords: effector memory $CD8^+$ T cells, NK-like T cells, natural killer receptors, innate receptors, open MHC-I conformers, IL-15, IFN-γ, tissue repair

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PREFACE

Thanks to their T cell antigen receptor (TCR), thymus-derived $CD8^+$ T cells have the unique ability to scrutinize any cell of our body displaying at the cell surface peptides bound to major histocompatibility class I (MHC-I) molecules and respond by means of cell activation and proliferation whenever the MHC-I molecule looks different than usual. In this regard, the quote "Divide and Conquer" (from the Latin saying *Divide et Impera*, credited to Julius Caesar) is the name of an algorithm that solves a problem by breaking it sequentially into two or more sub-problems until these become simple enough to be solved (1). Paraphrasing the quote and the algorithm, it can be anticipated that the tendency of a thymus-derived $CD8^+$ T cell to divide and generate a progeny of cells is meant to solve a problem, keep body homeostasis, by making conquerors that travel to distant injured/inflamed tissues (effector $CD8^+$ T cells), some of which may mutiny and become a problem (inflammatory $CD8^+$ T cells) and have to be restrained by peacekeepers (suppressor/regulator $CD8^+$ T cells). At the end of the process, a mixture of the different subsets survives as sensors of any further change that may occur within the environment they visited and conquered (memory $CD8^+$ T cells). Human $CD8^+$ T cell differentiation is a complex process enfolded in contrasting views on the functional role of the memory $CD8^+$ T cells under normal and diseased conditions. Hereby, we present a perspective on the function of these $CD8^+$ T cells that focus on the relationship with their internal environment.

CD8+ T CELL DIFFERENTIATION: ONE-WAY TICKET TO PLEIOTROPY

Before leaving the thymus to enter the circulation, CD8+ T cells survive two critical events that determine their fate in the periphery. First, they learn to *trans*-interact *via* their TCR clonotypic receptor with composites of a MHC-I heavy chain, a light chain (β_2m), and a short peptide (2). These antigen-presenting MHC-I structures are also designated "closed conformers" to distinguish them from the "open conformers" that are constituted only by the MHC-I heavy chain after dissociation from the light chain and/or the peptide and that can exist at the cell surface in an ordered non-denatured form (3). Open conformers can interact in *trans* and *cis* with a variety of receptors, namely members of the natural killer receptor (NKR) family, with important functional implications, as discussed below. The recognition of closed MHC-I conformers gives naïve CD8+ T cells the capacity to survive in the periphery and eventually recognize and be activated by closed MHC-I conformers presenting an excess of unusual antigens (4). After activation, naïve CD8+ T cells enter differentiation programs that result in the generation of effector CD8+ T cells displaying different bioactivities (5). After the excess of antigen is neutralized and removed, homeostatic mechanisms are turned on to cease the effector function while keeping a small pool that remains in circulation as memory CD8+ T cells (6). Second, CD8+ T cells are genetically programed to express an array of receptors during the differentiation process, which allows them to receive activation and survival signals from receptors and ligands other than MHC class I closed conformers (3, 7–10).

As a result of the huge effort done during the last decades and based on the expression of CCR7, CD27, CD28, CD45RA, and others, we have now a close picture of the main differentiation stages of human CD8+ T cells (**Figure 1**). Thus, the recirculating peripheral CD8+ T cell compartment is a mixture of lymphocytes distributed among five major pools: naïve (Tn), stem-cell memory (Tscm), central memory (Tcm), effector memory (Tem), and effector memory CD45RA+ (Temra) (11–13). An additional pool of non-recirculating tissue-resident memory cells (Trm) has also been described (14). Despite certain phenotypic and functional overlap among these CD8+ T cell pools, this classification has been most useful to describe the level of differentiation that the CD8+ T cell compartment has endured under different inflammatory settings, such as autoimmunity, cancer, and acute and chronic viral responses (15–17). Yet, perhaps the most significant achievement has been the identification of genes differently expressed by these pools, allowing to envision novel roles for CD8+ T cells (7, 18–20).

The CD8+ Tn pool comprises polyclonal T cells recently emigrated from the thymus that express CD28, CCR7, and CD62L, the two latter allowing them to recirculate between blood and secondary lymphoid organs (21). The CD8+ Tem and CD8+ Temra pools (for easiness, both termed as Tem thereafter) are highly differentiated CD8+ T cells that differ in the expression of the tyrosine phosphatase isoform CD45RA. They were formerly described as lymphocytes lacking CD28, responding poorly to TCR-stimulation, displaying redirected cytotoxicity, containing oligoclonal T cells, and being able to migrate to non-lymphoid organs and tissues (21–23). CD8+ T cells with the Tem phenotype were reported to express receptors thought to be solely expressed by NK cells, including CD56, CD94/NKG2A, killer Ig-like receptors (KIR), and leukocyte Ig-like receptors (LIR), among others (24–26). CD8+ T cells with the Tem phenotype also express NKp46 (27), a member of the natural cytotoxicity receptor, akin to several inhibitory receptors, such as CTLA-4, PD1, TIM3, and LAG3 (28). Due to these distinguishing features, they have also been designated CD8+CD28−, CD8+KIR+, CD8+NKR+, NK-like CD8+ T cells, and more recently innate CD8+ T cells (29–32). The evidence gathered during recent years suggests that the human CD8+ Tem pool is very diverse and polyfunctional and contains cells endowed with suppressor, inflammatory, and cytotoxic features (25–35). Whether these polyfunctional CD8+ Tem cells reflect the existence of distinctive subsets or a pleiotropic CD8+ T cell population that displays its activities depending on the signals that receive in the different tissue environments, remains to be elucidated.

ON THE ORIGIN OF NK-LIKE CD8+ T CELLS: AGING, VIRUSES, CYTOKINES, AND MORE

Following the initial description of CD8+CD28− T cells in the late 1980s and early 1990s, high levels of these cells were described in peripheral blood of healthy elderly people, during viral infections (e.g., CMV, HIV, and EBV), cancer, and autoimmunity (8). Nowadays, alterations in CD8+CD28− T cells have been reported in almost every chronic inflammatory disease. Studies performed on CMV-seropositive elderly showed that a sizable fraction of CD8+CD28− T cells contains CMV-specific CD8+ T cells (17). The description in the elderly of an association between the accumulation of CD8+CD28− T cells, a phenomenon called memory CD8+ T cell inflation (36), CMV seropositivity, a decrease in survival rate and faulty *in vivo* humoral and cellular responses to vaccination, brought about the view that CD8+ Tem cells were terminally differentiated dysfunctional cells that contributed to immunosenescence and susceptibility to develop chronic inflammatory diseases (35–40). Recent studies are revealing that CMV-specific CD8+ Tem are not dysfunctional cells. Rather, they are polyfunctional in terms of cytokine secretion and proliferation (41–44), capable of surviving for longer periods of time (45, 46), and are only functionally restricted by the set of inhibitory receptors they express (28, 41). On the other hand, longitudinal studies comparing IgG titers and DNA viral load with CMV-specific CD8+ T cell frequencies suggest that CMV serology may not be a reliable indicator to study associations between chronic CVM infection and CD8+ Tem cell expansions (47). Thus, the association between CD8+ T cell expansions, CMV seropositivity, immunosenescence, and predisposition to disease remains an open question (48).

Besides chronic activation by viral antigens, there is solid evidence that cytokines such as IL-15, TNF-α, and TGF-β, as well as several cell types, regulate CD8+ T cell homeostasis. IL-15 displays multiple bioactivities, namely induction and maintenance of CD8+ Tem cells *in vitro* and *in vivo* (49–54), suggesting that

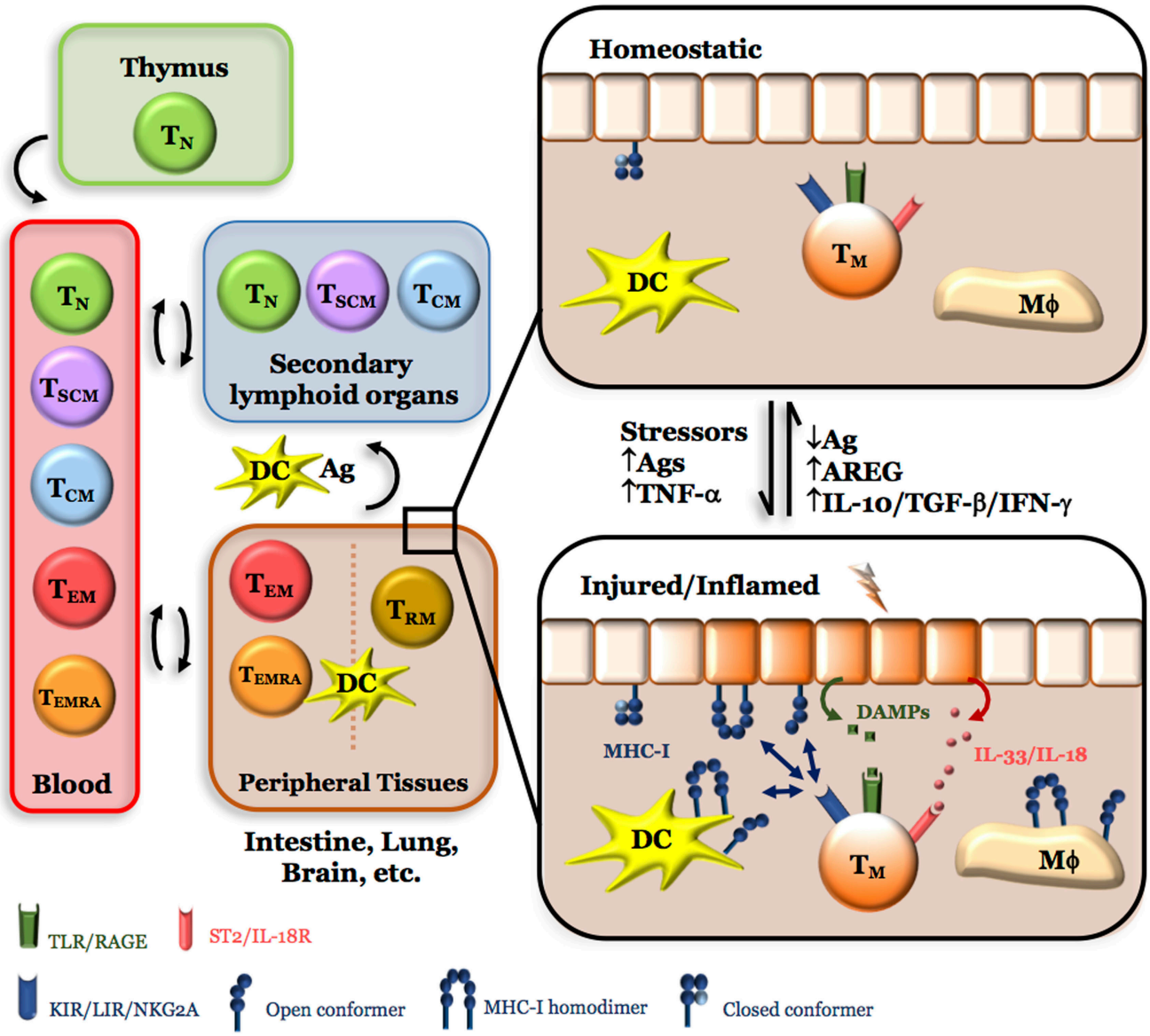

FIGURE 1 | Simplified model for the role of NK-like CD8+ TEM cells in tissue integrity. Of the five major circulating CD8+ T cell pools, naive (TN), stem-cell memory (TSCM), and central memory (TCM) preferentially migrate to secondary lymphoid organs, where they can be activated by processed antigens presented by closed major histocompatibility class I (MHC-I) conformers expressed by dendritic cells (DC) recently arrived from peripheral tissues and differentiate into effector memory (TEM) and effector memory CD45RA+ (TEMRA). On the other hand, CD8+ TEM and TEMRA have preferential, but not exclusive, access to peripheral tissues under homeostatic (healthy) conditions where they can stay as CD8+ TRM. Under tissue stress and/or injury, a sudden increase in antigens (Ags) and/or inflammatory cytokines (TNF-α) results in the release of endogenous products [damage-associated molecular patterns (DAMP), IL-33, ATP, etc.] and expression of open MHC-I conformers by immune and non-immune cells. While tissue DCs could migrate to secondary lymphoid organs and induce more cycles of CD8+ T cell activation and differentiation, TEM, TEMRA, and TRM (denoted as TM for simplicity) could directly sense these changes *in loco*; thanks to the expression of killer Ig-like receptor, leukocyte Ig-like receptor, NKG2A, DAMP receptors, IL-18/IL-33 receptors, purinergic receptors, and others. Thus, the presence of CD8+ TM cells in peripheral tissues allows a faster response to harmful situations by secreting cytokines (IFN-γ, IL-10, TGF-β) and factors [amphiregulin (AREG)] that activate pathways leading to tissue repair and regeneration, and, therefore, to the homeostatic (healthy) state. Any imbalance in this equilibrium (e.g., overt tissue injury and necrosis, hypoxia, excess of antigen, high CD4/CD8 T cell ratios, low numbers or absence of CD8+ TEM, TEMRA, TRM cells, etc.) will result in a failure to resolve inflammation and chronic inflammation will ensue.

memory CD8+ T cell inflation may also result from encounters with cytokines, thus increasing virtual memory CD8+ T cells (55). IL-15 is also involved in liver homeostasis and regeneration after hepatectomy (56). Whether this bioactivity is linked to the pro-survival activities of hepatocytes on CD8+ T cells and the presence of large amounts of CD8+ TEM cells in the liver, remains to be elucidated (27, 57). On the other hand, reconstitution studies in mice have shown that accumulation of CD8+ TEM cells

depends on the presence of IL-15 and CD4+ T cells (9). Finally, intestinal epithelial cells and 4-1BBL+ B cells have also been shown to drive expansion and accumulation of CD8+ T cells with the Tem phenotype *in vitro* (58, 59) and *in vivo* (60), respectively, expanding the universe of factors that drive NK-like CD8+ T cell generation.

While unnoticed, expansions of CD8+ T cells with a Tem phenotype were described in conditions where oxidative stress is high, including HFE hemochromatosis, heavy alcohol consumption, hemodialysis, β-thalassemia, and during acute exercise (61–65). Although the molecular cues underlying CD8+CD28− Tem generation under stressful conditions are uncertain, PGE2, a byproduct of arachidonic acid catabolism produced under pro-oxidant and inflammatory conditions, induces expression of NKG2A and downregulates CD28 on CD8+ T cells, two features associated with the acquisition of the Tem phenotype (66–68). Oxidative stress has also been shown to regulate expression of Bach2, a transcription factor involved in the formation of CD8+ Tem cells through downregulation of genes associated with effector function, such as Blimp1 (69, 70). On the other hand, expression of the transcription factor HIF-1 by CD8+ T cells *in vitro* under low oxygen conditions, mimicking acute exercise, correlates with accumulation of CD8+ Tem cells (71), which is in agreement with the reported role of HFI-1 in modulating the balance between effector and memory CD8+ T cells in models of chronic activation (72). These data suggest that oxidative stress may play an important role in modulating the formation of CD8+ Tem cells.

Finally, the CD4/CD8 T cell ratio is a factor that appears to influence the extent of the CD8+CD28− T cell expansions. Early studies in HFE hemochromatosis and heavy alcohol drinkers reported a positive correlation between the size of the CD8+CD28− T cell expansions and the size of the CD8+ T cell pool, regardless of age (61, 62). Importantly, the regression curve had a much higher slope and correlation coefficient in the patients than in the control group, implying that under stressful/adverse conditions there is a hastened formation of CD8+CD28− T cells (8, 61, 62). Similar results were observed when the percentage of CD8+CD56+ T cells, which is increased in the elderly, was analyzed (73), strongly suggesting that the expansions of CD8+CD28− T cells are constrained by the size of the CD8+ T cell pool in relation to the CD4+, which are both under the control of major autosomal recessive genes (74). The importance of this influence is illustrated by two sets of studies. First, studies in infants with overt CD4+ T cell lymphopenia and reversed CD4/CD8 T cell ratios, due to deficiency in the tyrosine kinase p56lck, showed that the peripheral CD8+ T cell pool was made up almost entirely of CD8+ T cells with the Tem phenotype (75–77). Second, a recent cross-sectional study in elderly people, including centenarians, showed that the heterogeneity found in the CD8+ Tem pool could be explained by variations in the size of the CD8+ T cell compartment (78). Although it is difficult to discern what is cause and what is effect, we favor the view that the CD4/CD8 T cell ratio influences the extent of the CD8+CD28− T cell expansions. In this context, it is worth mentioning that the expansions CD8+CD28− T cells reported in HFE hemochromatosis patients were paralleled by a defective CD8-associated p56lck (61, 79). In view of these data and studies in mice showing that CD8-associated Lck is dispensable for maintenance of memory CD8+ T cells (80), it is tempting to speculate that in humans a deficient CD8-p56lck signaling and expansion of CD8+CD28− T cells could be intertwined processes.

ON THE FUNCTION OF NK-LIKE CD8+ T CELLS: THERE IS LIFE BEYOND CLOSED MHC-I CONFORMERS

The accumulated evidence indicates that loss of CD28, shrinkage of the TCR repertoire, gain of a variety of NKR, and expression of tissue homing receptors are interdependent events that end up in the formation of polyfunctional human CD8+ Tem cells that migrate to peripheral tissues where a fraction stays as a pool of non-recirculating CD8+ Trm cells upon expression of CD69 and CD103 (14). A series of recent studies using tissue samples from otherwise healthy infant and adult organ donors have shown that CD8+ Tem cells are predominant within non-lymphoid tissues and organs, including the brain, and this prevalence increases from childhood to adultness (81–84). CD8+ Tem predominance also occurs in the healthy bone marrow, stomach, and gingiva (85–87). Interestingly, lower CD4/CD8 T cell ratios within these tissues are associated with a larger CD8+ Tem pool (81), pointing again to the importance of the molecular cues that regulate this setting. Although recirculating and non-recirculating CD8+ Tem present in non-lymphoid tissues confer local immune protection against infections (88–90), it is also true that CD8+ Tem cells adapt to the new environment and may participate in the resolution of inflammation followed by tissue regeneration and repair after injury through complex networks involving cross-talk with other tissue environmental cells (91–99).

The picture emerges where CD28 loss and expression KIR, LIR and other NKR allows CD8+ Tem cells to engage in a cross-talk with other cells in their environment (8, 100, 101). But how this acquired skill is conveyed in terms of control of tissue integrity and organ function? As already mentioned, cell surface MHC-I molecules can exist in equilibrium between closed and open conformers, a process that is regulated by endocytosis and phosphorylation of a conserved tyrosine residue in the cytoplasmic tail of MHC-I heavy chains, and that allows the open conformers to self-associate and form novel structures called class I homodimers (3, 102–104). In this context, a series of recent reports are unveiling the many lives of MHC class I molecules (105), by showing that besides interacting with closed conformers in a peptide-independent manner (106, 107), KIR and LIR also interact with open conformers and homodimers (108–114). These results are of upmost importance if we consider that open conformers are expressed and/or released by metabolically active and stressed immune and non-immune cells, including neurons (103, 115–119). Thus, expression of NKR by CD8+ Tem cells allows them to sense changes in the level of closed and open MHC-I conformers, i.e., in the stressful/inflammatory state of the environment. The impact that KIR/LIR/NKG2A engagement has on CD8+ T cell survival and cytokine secretion (29, 52, 120), is of the foremost importance

for tissue homeostasis under normal or pathological conditions. Thus, IFN-γ and other cytokines released by CD8+ Tem upon NKR triggering could mediate resolution of inflammation and subsequent tissue healing by modulating growth and proliferation of epithelial and other tissue cells (121, 122). Importantly, by inducing upregulation of classical and non-classical MHC-I molecules on epithelial/endothelial cells, IFN-γ may further promote survival and proliferation signals upon MHC-I reverse signaling by their cognate NKR expressed by CD8+ Tem cells (123), thus harnessing the healing process.

Although expression of NKR allows CD8+ Tem cells located in peripheral tissues to sense changes in the closed ↔ open MHC-I equilibrium, this is likely not enough to cope with the fluctuations that occur within an ever changing internal environment (**Figure 1**). In this respect, there is evidence that CD8+ Tem cells also express receptors for damage-associated molecular patterns (DAMP), which are specialized in recognizing endogenous products released by cell stress, injury, or dead (124). DAMP receptors include toll-like receptors (TLR), advance glycosylation end products receptors (RAGE), receptors for IL-1 family members (e.g., IL-18 and IL-33), purinergic receptors (P2YR), and β2-adrenergic receptors (19, 125–129), to cite some. Although most of the studies on these receptors have focused on innate cells, there is growing evidence that DAMP receptor expression by CD8+ Tem cells could broaden their capacity to sense the disruption of tissue homeostasis and respond by secreting regulatory cytokines and healing factors. Thus, ligation of TLR on CD8+ Tem cells is known to augment IFN-γ in *in vitro* and *in vivo* settings (130, 131), which in barrier tissues such as the lung, where a large fraction of CD8+ T cells are Temra (81) may exacerbate tissue pathology (132). RAGE encompasses multiple ligands, including glycated proteins, nuclear high-mobility group box 1 (HMGB1), S100 proteins, and β-amyloid, among others, which transmit intracellular signals associated with tissue repair and regeneration (133, 134). RAGE+CD8+ T cells were described more than two decades ago and proposed to participate in the regulation of tissue homeostasis through secretion of IFN-γ (135). HMGB1 can also bind to TIM3, an inhibitory receptor expressed on CD8+ Temra cells, whose inhibitory function depends on the co-expression of CEACAM-1 (28, 136). Although expression of the IL-18 and IL-33 receptors by CD8+ T cells is known for some time (127), their importance in the regulation of tissue repair by T cells has only recently emerged (137–139). Thus, binding of IL-18 and IL-33 to regulatory T cells triggers the secretion of amphiregulin (AREG), a ligand for the EGF receptor involved in suppression of inflammation and tissue repair (140, 141). Although formal proof for the secretion of AREG by CD8+ Tem cells is lacking, there is evidence that CD8+ T cells express this tissue repair factor (141, 142). Finally, CD8+ Tem cells also express purinergic receptors (19), as well as the β2-adrenergic receptor (129). While the former can sense environmental nucleotides released under adverse conditions and induce suppressive signals on T cells, thus downplaying inflammation (128, 143), the latter allows the sympathetic nervous system to communicate under stressful conditions with CD8+ T cells (144, 145).

CONCLUDING REMARKS

Although one important facet of CD8+ T cells has to do with tissue damage and injury resulting from coping with infections, this should not overshadow other facets of CD8+ T cells related with the maintenance of tissue integrity and homeostasis (**Figure 1**). Since the description of CD8+CD28− T cells about 25 years ago, a huge amount of information has been obtained on the functional phenotype and localization of these lymphocytes. Their capacity to migrate and reside in peripheral tissues in parallel with the expression of receptors for unconventional MHC-I molecules and endogenous products released by injured, inflamed, and necrotic cells may endow these cells with the capacity to fine-tune tissue repair, regeneration, and homeostasis by a number of ways, namely by inducing epithelial, endothelial, and mesenchymal cells to grow and proliferate and inhibiting inflammatory responses. All these bioactivities will likely involve active crosstalk with environmental cells and complex loops between secreted cytokines.

AUTHOR CONTRIBUTIONS

FA and EC wrote the manuscript. EC drew the figure. AE and CP read and edited the manuscript.

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OPINION
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CD8+ T Cells in Chronic Periodontitis: Roles and Rules

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INTRODUCTION

Chronic periodontitis is a multifactorial disease characterized by the presence of dysbiotic microbial communities that, together with genetic and environmental factors, results in chronic inflammation of the periodontium, which ultimately may trigger alveolar bone resorption (1). The reported associations between periodontal disease and other chronic disorders, including metabolic, cardiovascular, respiratory, and arthritic diseases (2), reinforce the importance of elucidating the cell types and molecular mechanisms involved. Chronic inflammation of gingival tissue has long been associated with infiltration of the gingiva by activated T and B cells and secretion of inflammatory cytokines and immunoglobulins. However, the role of T cells in periodontal disease is controversial with reports showing that they have both protective and destructive roles (3, 4). Moreover, while most studies have focused on CD4+ T cells and B cells, the role played by gingival CD8+ T cells has been overlooked. On the basis of data published recently, we discuss the possibility that gingival CD8+ T cells contain a pool of cells with regulatory/suppressor properties involved in the maintenance of gingival tissue integrity by constitutively downregulating inflammation under homeostatic conditions and initiating repairing mechanisms in case of tissue injury. These basic physiological roles could be surpassed and hidden when a potent and/or chronic immune response against pathogenic bacterial colonization occurs, thus leading to bone loss. Elucidation of the basic physiological roles of particular CD8+ T cells present in periodontal tissue and the rules they follow in order to cope with minor versus major disruption of tissue homeostasis can improve our understanding of how they react to changes in their environment and ultimately allow the development of novel therapeutic approaches to favor anti-inflammatory responses and bone repair.

THE ROLES OF CD8+ T CELLS: CYTOTOXICITY, SUPPRESSION, AND TISSUE REPAIR

One fascinating aspect of CD8+ T cells is their heterogeneity, as they differ in terms of T cell receptor (TCR) diversity and antigen specificity, which allows them to monitor for shifts in peptide antigens presented in MHC class I molecules expressed on the plasma membrane of all nucleated cells. CD8+ T cells acquire functional properties after being activated, normally in secondary lymphoid organs, by antigen presenting cells (APC). As a result, some acquire innate receptors, including NK receptors, enlarging the kind of stimuli they can receive (5). In addition to the well-documented cytotoxic activities, CD8+ T cells might also have regulatory/suppressor functions (thereafter CD8+ Treg) since they have the ability to control other leukocytes to avoid excessive immune activation and its pathological consequences (6, 7). Although in humans many phenotypes have been described for CD8+ Treg, the most reliable maker is the transcription factor Foxp3 (7). Nevertheless, CD8+ Tregs have also been described as Foxp3^{low} (8) and Foxp3−, including CD8^{low}CD28−, CD8+CD103+, and non-antigen-specific CD8+CD28− (9–11). CD8+ Tregs are also diverse regarding the mechanisms of suppression, which include induction

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of tolerogenic APC, withdrawal of homeostatic cytokines, secretion of anti-inflammatory cytokines, and cell cytotoxicity (6, 7). The importance of $CD8^+$ Treg in the context of tissue inflammation is illustrated by a series of studies showing that interactions between intestinal epithelial cells and $CD8^+$ T cells induce a population of $CD8^+CD28^-CD103^+$ T cells endowed with suppressor functions (12, 13). Subsequent studies by the same group demonstrated that the disruption of their suppressor activities is associated with mucosal inflammation (8). The regulatory functions of highly differentiated $CD8^+$ T cells might also include tissue repair (14), although it has been mainly described in $CD4^+$ Treg for lung (15), and αβ and γδ T cells for bone (16, 17). Indeed, the experimental evidence for the existence of $CD8^+$ T cells endowed with tissue repair and/or bone regeneration properties within the gingival tissue is very scarce as described below, which warrants further studies.

$CD8^+$ T CELLS AND BONE HOMEOSTASIS: CYTOKINES MATTER

An early seminal study using NOD/SCID mice transplanted with human peripheral blood lymphocytes as a model of periodontitis showed that human $CD4^+$ T cells in the periodontium triggered local alveolar bone destruction by secreting osteoprotegerin ligand, also known as RANKL (18). Subsequently, it was demonstrated that several pro-inflammatory cytokines, for example TNF-α, ultimately converge on the expression of RANKL thus promoting osteoclastogenesis (19). In contrast, anti-inflammatory cytokines secreted by $CD8^+$ T cell with an effector–memory phenotype, such as IL-10 and TGF-β (7, 20), have been shown to be bone protective in *in vivo* and *in vitro* models of bone regeneration (19, 21, 22). The role of pro-inflammatory cytokines IL-17 and IFN-γ in bone homeostasis remains controversial. IL-17 is mainly produced by $CD4^+$ Th17 cells and γδ T cells and has been associated with bone destruction (23). However, recent experimental studies in knockout mice models have shown that IL-17 may participate in the early phases of bone regeneration by directly stimulating osteoblastogenesis (16, 17). Regarding IFN-γ, it has been shown to promote as well as to inhibit bone formation (23–25). The contrasting effects of IFN-γ could perhaps be explained by the fact that it may exert a direct inhibitory effect on osteoclastogenesis by interfering with the RANK pathway, and at the same time promote bone destruction indirectly by inducing antigen-presenting MHC molecules on APC, leading to increased production of TNF-α by activated T cells (26). Whether the IFN-γ discrepancies on bone homeostasis may result from the study of early versus late phases of bone formation, as it happens with IL-17, remain to be elucidated. Importantly, recent studies in models of bone regeneration have shown that mouse $CD8^+$ T cells and *in vitro* expanded human $CD8^+$ T cells secrete Wnt10b, a cytokine/factor that promotes osteoblastogenesis (27). Even though the exact mechanism used by $CD8^+$ T cells to promote bone regeneration remains to be elucidated, the accumulated evidence from experimental models of bone regeneration suggests that cytokines and factors secreted by $CD8^+$ T cells could be involved.

GINGIVAL $CD8^+$ T CELLS AND CHRONIC PERIODONTITIS

The majority of studies on gingival tissue of chronic periodontitis focused on the functional characterization of $CD4^+$ T cells and B cells and concluded that the presence of $CD4^+$ Th1 cells and antibody-secreting B cells, as a result of the host immune response against bacterial infection, was associated with chronic inflammation and disease progression, namely alveolar bone loss (1). Indeed, a detrimental role of gingival $CD4^+$ T cells in alveolar bone destruction under chronic periodontitis has been steadily proposed since the seminal work of Penninger's group (18). These conclusions are supported by an *in vivo* study in CD4- and CD8-deficient mice showing that $CD4^+$ T cells contribute to the alveolar bone loss in mice (28). Although $CD4^+$ Treg cells have been shown to confer protection in animal models of periodontal disease (29, 30), they are present in low numbers in gingival tissue of subjects with chronic periodontal disease (31, 32) and some of them may switch to an inflammatory Th17 phenotype (33).

Although the role of $CD8^+$ T cells in chronic periodontitis is less obvious, most studies have consistently shown that despite being more abundant in gingival tissues of periodontitis patients than in patients with gingivitis or healthy controls, $CD8^+$ T cells are not involved in gingival tissue pathology (3, 4). Similar conclusions were drawn from mice studies (28). Interestingly, a recent comprehensive study using multiparametric flow cytometry has revealed that T cells present in healthy gingival tissue are predominantly effector–memory, as determined by the use of CD45RA, CD45RO, and CCR7, with $CD4^+$ and $CD8^+$ T cells being more abundant than B cells (34). In gingival tissue from chronic periodontitis, a marked increase in total $CD4^+$ T cells, $CD8^+$ T cells, and B cells, akin to neutrophils, was observed, and most of the $CD4^+$ T cells produced IL-17 (34). Although in the study of Dutzan et al. $CD8^+$ Treg are not detected in the gingiva of chronic periodontitis patients (34), previous studies showed that some gingival $CD8^+$ T cells lack CD28 while expressing the inhibitory receptor PD1 (35–37). These features are associated with an effector–memory phenotype (20). These data, together with early *in vitro* studies showing that $CD8^+$ T cells confer bone protection by suppressing osteoclastogenesis (38, 39), suggest that effector–memory $CD8^+$ T cells present in gingival tissue might play a key basic physiological role in safeguarding tissue integrity (**Figure 1**). Nevertheless, further studies are needed to ascribe a particular protective role to specific $CD8^+$ T cells. Indeed, this role may be overwhelmed and masked by inflated T and B cell immune responses against bacterial aggression under dysbiotic bacterial growth, with dramatic outcomes for bone homeostasis.

GINGIVAL $CD8^+$ T CELLS: MOLECULAR SIGNALS AND EFFECTOR FUNCTIONS

Given the effector–memory phenotype (34–37), and unlike $CD4^+$ T cells that express CD28 and may respond to TCR/CD28-mediated signals, gingival $CD8^+$ T cells may preferentially

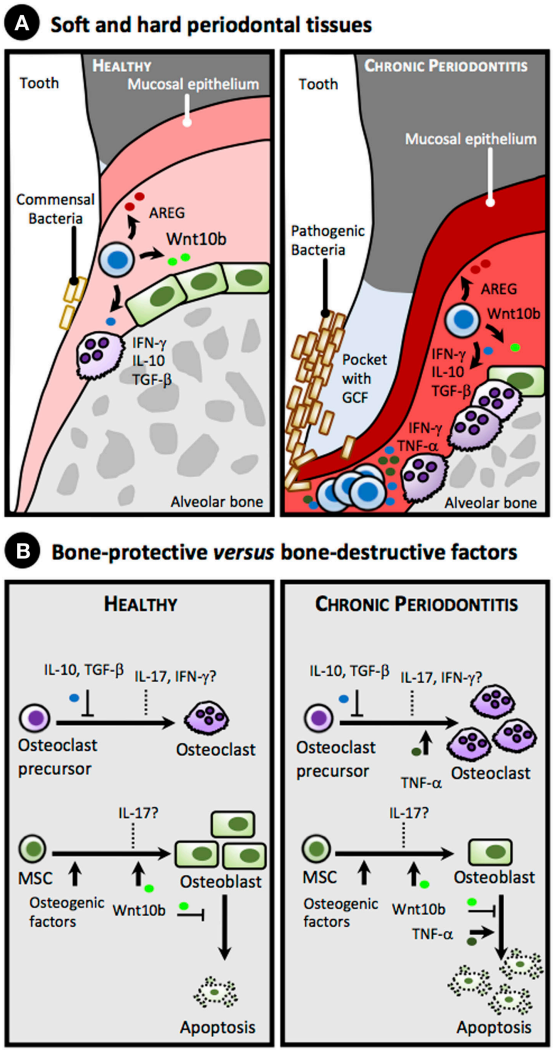

FIGURE 1 | Proposed simplified model for the role of gingival CD8+ T cell-derived cytokines and factors in alveolar bone homeostasis in health and chronic periodontitis. (A) Anatomy of soft and hard periodontal tissues showing selected cells: CD8+ T cells, herein collectively referred as memory CD8+ T cells or T_M (blue), osteoblasts (green), and osteoclasts (purple), in healthy periodontal tissues (left panel) and in chronic periodontitis (right panel). Note accumulation of pathogenic bacteria on the tooth surface, formation of a pocket with gingival crevicular fluid (GCF) accumulation, gingival attachment loss, inflamed gingiva (reddish), as well as loss of alveolar bone in chronic periodontitis due to imbalance in the equilibrium between osteoblastogenesis and osteoclastogenesis toward the latter (right panel). **(B)** Bone-protective versus bone-destructive factors that can be produced by gingival CD8+ T_M cells. Under homeostatic conditions (left panel), IL-10 and TGF-β secreted by CD8+ T_M cells suppress osteoclastogenesis, while Wntb10 promotes differentiation of mesenchymal stem cells (MSC) into osteoblasts and inhibits their apoptosis. In addition, CD8+ T_M cells secrete amphiregulin (AREG) that downplays gingival inflammation and promotes epithelial and stromal tissue repair (data not shown, see text). Overall, these bone promoting cytokines counteract the bone loss induced by commensal bacteria (see text). The role of IFN-γ and IL-17 under homeostatic conditions on osteoclastogenesis and osteoblastogenesis is uncertain (dashed lines). Under chronic periodontitis (right panel) high levels of TNF-α, and perhaps of IFN-γ and IL-17, produced in response to pathogenic bacterial colonization surpass the protective role of the aforementioned cytokines, promoting osteoclastogenesis and increasing apoptosis of osteoblasts, thus favoring bone loss.

be activated in a TCR-independent manner by local signals produced during stress and/or injury, including a variety of endogenous products that signal through innate receptors, as discussed elsewhere (20). As a result, upon innate receptor triggering gingival CD8+ T cells may secrete cytokines, such as IL-10 and IFN-γ, reported to have bone repairing properties (23, 26). Interestingly, a recent study showed that, unlike inflammatory cytokines, the levels of IL-10 remained unchanged in the gingival crevicular fluid of chronic periodontitis patients after non-surgical periodontal therapy (40), suggesting that IL-10 may have indeed a basic physiologic role in the healthy gingiva, as demonstrated in IL-10-deficient mice (41). In addition, gingival CD8+ T cells could further improve tissue healing after receiving environmental signals by secreting amphiregulin, an anti-inflammatory cytokine expressed by CD8+ T cells (42), which has been shown to promote tissue repair (14), and is upregulated in the gingival stroma in a mice model of chronic periodontitis (43) (**Figure 1**).

Evidence for the expression of innate/inhibitory receptors by gingival CD8+ T cells, including KIR, LIR, TLR, and others, is very scarce, which warrants the need and importance of studying their expression. Thus, though initially considered a T cell exhaustion marker, the reported expression of PD1 by gingival CD8+ T cells (37) could potentially be involved in limiting tissue damage through interaction with its ligand, which can be expressed by a variety of stromal cells (44, 45). On the other hand, *in vitro* studies in mice have recently proposed that CD8+ T cells could be activated by osteoclasts *via* antigen cross-presentation, resulting in the formation of CD8+ Treg that could inhibit bone resorption through secretion of IFN-γ (46). These results are challenging and suggest that bone-protective CD8+ T cells could be generated *in loco* from resident CD8+ T cells in the alveolar bone surface, while bone-protective CD4+ Treg may be recruited from circulation (29, 30). In this respect, it is important to mention that the commensal bacteria present in the gingiva may exert an important role in alveolar bone homeostasis. Thus, a series of animal studies performed in germ-free *versus* specific pathogen-free *versus* wild-type models have shown that commensal bacteria present in the gingiva is responsible for physiological alveolar bone loss (1, 47), suggesting that gingival T cells and their secreted cytokines might be present from birth, as seen in other peripheral tissues (48), and contribute to physiologic alveolar bone homeostasis in healthy conditions.

CONCLUDING REMARKS AND FUTURE PROSPECTS

While scant, there is evidence that resident gingival CD8+ T cells may contain lymphocytes with regulatory functions, including suppression of bone-destructive cytokines and repair of alveolar bone, two activities that could be intertwined. However, the host immune response that takes place upon chronic bacterial colonization of the teeth and that results in the recruitment of innate and adaptive inflammatory cells (34) will likely disrupt these homeostatic activities. Thus, it turns out of the foremost importance to elucidate the role of gingival effector–memory CD8+ T cells in bone remodeling in health and sickly conditions. To do that, the

use of mice models lacking selected populations (cytotoxic CD8+ T cells versus CD8+ Treg, etc.), suppressive cytokines or tissue-specific chemokine receptors, or adoptive transfer of CD8+ Treg should provide insights into the bone-protective role of gingival CD8+ T cells (30, 49, 50). These studies will certainly broaden our understanding of the relationship of gingival CD8+ T cells with the periodontium and enable the development of novel therapies to inhibit bone loss under pathological conditions.

AUTHOR CONTRIBUTIONS

EC did bibliography search and wrote the manuscript. FA did bibliography search and edited the manuscript.

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MINI REVIEW
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Steroid Resistant CD8$^+$CD28^{null} NKT-Like Pro-inflammatory Cytotoxic Cells in Chronic Obstructive Pulmonary Disease

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Corticosteroid resistance is a major barrier to effective treatment in chronic obstructive pulmonary disease (COPD), and failure to suppress systemic inflammation in these patients may result in increased comorbidity. Although much of the research to date has focused on the role of macrophages and neutrophils involved in inflammation in the airways in COPD, recent evidence suggests that CD8$^+$ T cells may be central regulators of the inflammatory network in this disease. CD8$^+$ cytotoxic pro-inflammatory T cells have been shown to be increased in the peripheral blood and airways in patients with COPD, whereas smokers that have not progressed to COPD only show an increase in the lungs. Although the mechanisms underlying steroid resistance in these lymphocytes is largely unknown, new research has identified a role for cytotoxic pro-inflammatory CD8$^+$ T-cells and CD8$^+$ natural killer T-like (NKT-like) cells. Increased numbers of these cells and their significant loss of the co-stimulatory molecule CD28 have been shown in COPD, consistent with findings in the elderly and in clinical conditions involving chronic activation of the immune system. In COPD, these senescent cells expressed increased levels of the cytotoxic mediators, perforin and granzyme b, and the pro-inflammatory cytokines, IFNγ and TNFα. They also demonstrated increased cytotoxicity toward lung epithelial cells and importantly were resistant to immunosuppression by corticosteroids compared with their CD28$^+$ counterparts. Further research has shown these cells evade the immunosuppressive effects of steroids *via* multiple mechanisms. This mini review will focus on cytotoxic pro-inflammatory CD8$^+$CD28^{null} NKT-like cells involved in COPD and novel approaches to reverse steroid resistance in these cells.

Keywords: CD8$^+$ NKT-like cell, steroid resistance, chronic obstructive pulmonary disease, CD28, IFNγ and TNFα, Pgp, HDAC2, Hsp90

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CD8$^+$ NATURAL KILLER T-LIKE (NKT-LIKE) CELLS IN CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD)

Natural killer T-like cells comprise a unique subgroup of lymphocytes that express features of both T cells and natural killer (NK) cells. NKT-like cells co-express T-cell receptors and CD4 or CD8 (or CD4$^-$/CD8$^-$), together with markers associated with NK cells, such as CD56 (**Figure 1C**) and/or CD16 or CD161. Acquisition of CD11b represents an early event in CD8$^+$ T-cell differentiation, which may allow extravasation to peripheral tissues (1, 2). These cells are a small but important

subset of lymphocytes that represent a bridge between innate and adaptive immunity.

There has been conflicting evidence regarding changes in NKT-like cell numbers in COPD. Numbers of these cells have been reported to be decreased in the peripheral blood of patients with COPD (3). One study showed numbers to be unchanged (4), while a third reported increased numbers (5). However, further characterization into $CD4^+$ or $CD8^+$ NKT-like cells was not performed in any of these reports. NKT-like cells have also been reported to be increased in induced sputum and bronchoalveolar lavage (BAL) of COPD patients and, importantly, have been shown to be cytotoxic to autologous lung cells (3, 4, 6).

LOSS OF CD28 ON SENESCENT LYMPHOCYTES IN COPD

Following persistent antigenic stimulation, NKT-like cells can lose co-stimulatory molecules, undergo telomere shortening, and exhibit defective IL-2 production; changes that define the state of replicative senescence. The majority of these "effector senescent" lymphocytes are $CD8^+$, $CD45RA^+$, $CD11a^{bright}$, $CD28^{null}$ (**Figure 1D**), $CD62L^-$, and $CCR7^-$. Expansion of these cells are found in the elderly and in other clinical conditions involving chronic activation of the immune system such as viral infections, rheumatic, and autoimmune diseases (7). Increased numbers have also been reported in chronic inflammatory lung diseases including COPD and in patients following lung transplantation (8, 9).

STEROID RESISTANCE IN $CD8^+CD28^{null}$ NKT-LIKE CELLS IN COPD

Steroid Resistant $CD8^+$ T Cells in COPD

Patients with COPD have been shown to be resistant to the immunosuppressant effects or glucocorticoids (10). Most of the investigations into steroid resistance in this disease have focused on the role of the airway macrophages and neutrophils (10); however,

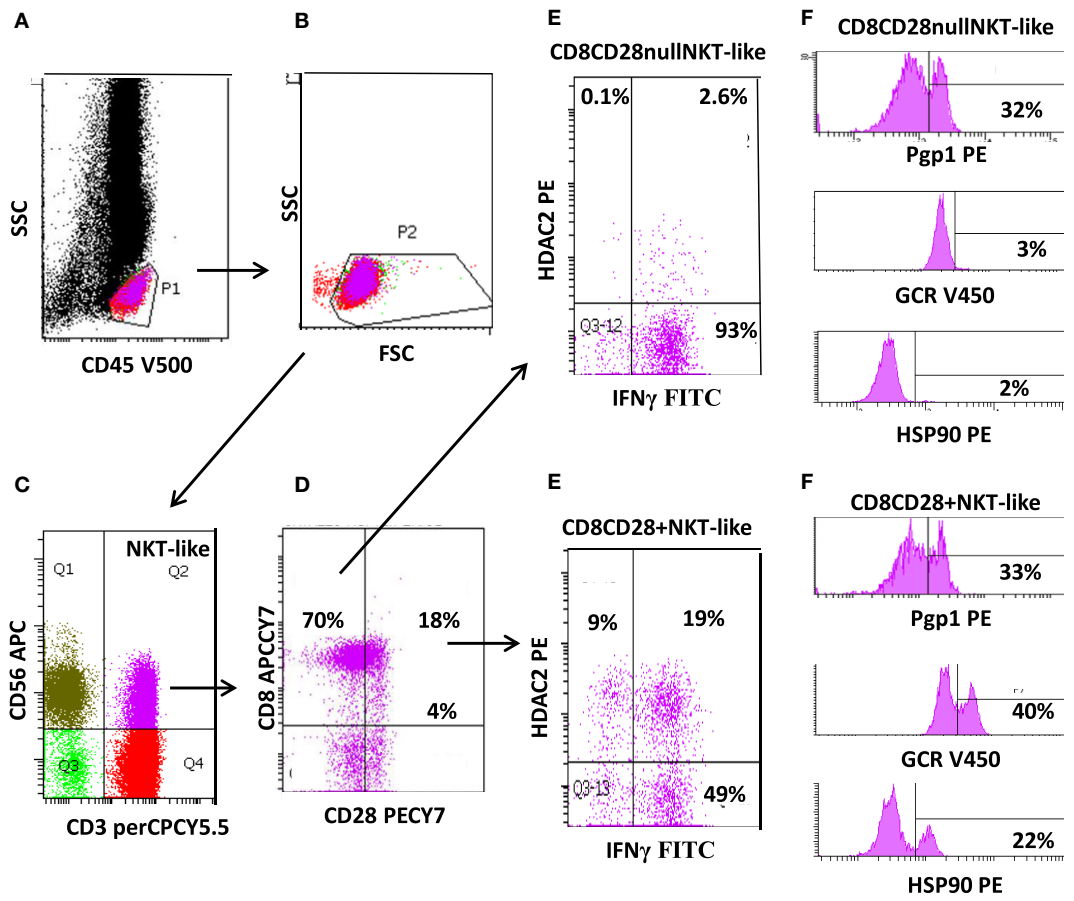

FIGURE 1 | Flow cytometric gating technique used to identify $CD8^+CD28^{null}$ natural killer T-like (NKT-like) cells (and $CD8^+CD28^+$ NKT-like cells) from the peripheral blood of patients with chronic obstructive pulmonary disease. **(A)** Identification of lymphocytes as $CD45^+$ low side scatter (SSC) events. **(B)** Removal of red blood cell contaminations removed by lymphocyte gating using forward scatter vs. SSC characteristics. **(C)** Identification of NKT-like cells as $CD3^+CD56^+$ events. **(D)** Identification of $CD28^{null}$ and $CD28^+$ NKT-like cells using CD8 vs. CD28 staining. **(E)** Expression of IFNγ and histone deacetylase (HDAC)2 in $CD8^+CD28^{null}$ and $CD8^+CD28^+$ cells. **(F)** Expression of P-glycoprotein-1 (Pgp1), glucocorticoid receptor (GCR), and heat shock protein (Hsp)90 expression in $CD8^+CD28^{null}$ and $CD8^+CD28^+$ cells. Note: the majority of NKT-like cells are $CD8^+$ and $CD28^{null}$. These cells express reduced HDAC2, GCR, and Hsp90 but increased IFNγ compared with $CD8^+CD28^+$ NKT-like cells (Pgp1 unchanged).

the mechanisms underlying steroid resistance in lymphocytes in patients with COPD until recently has been largely unknown. The role of T-cells is likely to be important in this regard, as their increased numbers have been reported in the lungs of patients with COPD. A study by Maeno et al. demonstrated an important requirement for $CD8^+$ T cells in the development of cigarette smoke-induced emphysema. They suggested a unifying pathway whereby $CD8^+$ T cells are the central regulators of the inflammation network in COPD (11). Inhaled corticosteroids have been shown to reduce exacerbation rates and improve health status in patients with COPD but can also increase the risk of pneumonia (12, 13). The numbers of bronchial $CD8^+$ T-cells were reduced following long-term treatment with inhaled corticosteroids in ex-smoker COPD patients only but not persistent COPD smokers (12, 13).

There have been reports of increased numbers of $CD8^+$ T cells in the peripheral blood, BAL, and lung parenchyma from COPD smoker and ex-smoker patients compared with healthy smokers and control subjects (14, 15). This indicates the systemic involvement of these cells in COPD. The production of the pro-inflammatory cytokines, IFNγ and TNFα, by $CD8^+$ T cells was increased from peripheral blood, BAL, and intraepithelial compartments in patients with COPD. This was regardless of whether patients were receiving inhaled corticosteroids (14) indicating the lack of effectiveness of steroids at reducing pro-inflammatory cytokines by these cells. However, further lymphocyte subtyping with NKT-like cell markers was not performed. Steroid resistance was further shown *in vitro* by assessing the production of IFNγ by follicular $CD8^+$ T cells in the presence of 0.1–1μM dexamethasome (16), although further subtyping of NKT-like subsets was not performed in the study. Recently, steroid resistant $CD8^+CD28^{null}$ NKT-like cells were reported to be increased in number and to express increased levels of the cytotoxic mediators, perforin and granzyme b. Pro-inflammatory cytokines, IFNγ and TNFα (8), were also increased in the peripheral blood of patients with COPD, confirming the important role of these lymphocytes in steroid resistance.

P-glycoprotein-1 (Pgp1) in $CD8^+CD28^{null}$ NKT-Like Cells

P-glycoprotein is a transmembrane efflux pump well-characterized in drug-resistant cancer cells (17) and also thought to play a role in the function of steroid resistant lymphocytes in COPD. Pgp1 expression has been shown to be increased in T, NKT, and NK cells that also co-express IFNγ, TNFα, and granzyme b, in peripheral blood from COPD patients compared with healthy controls (**Figure 1**). However, further differentiation of NKT-like cells into $CD4^+$ and $CD8^+$ subsets was not performed (18).

Recent further investigations by the same authors comparing COPD patients with healthy age-matched controls showed no difference in Pgp1 expression between $CD8^+CD28^{null}$ NKT-like and $CD28^+CD8^+$ NKT-like subsets. However, the percentages of $CD8^+Pgp1^+CD28^{null}$ NKT-like and $CD8^+Pgp1^+CD28^+$ NKT-like cells were both increased in the COPD group (8) (**Figure 2A**). Treatment with very low-dose cyclosporine A (CsA), a Pgp1 inhibitor (2.5 ng/ml; approximately 25 times less than that used for transplant rejection therapy), combined with standard dose corticosteroid [1μM prednisolone (pred)] resulted in synergistic inhibition of pro-inflammatory cytokines in $CD8^+Pgp1^+CD28^{null}$ NKT-like cells (18) (**Figure 2B**). These data indicate that these agents may be an effective add-on therapy to standard steroid treatment.

Loss of Glucocorticoid Receptor (GCR) in $CD8^+CD28^{null}$ NKT-Like Cells in COPD

Glucocorticoids must bind to the GCR in the cytoplasm of a cell before being transported to the nucleus. A recent study examined the expression of GCR in pro-inflammatory NKT-like cells in the peripheral blood of patients with COPD (8). COPD was associated with increased percentage of $CD28^{null}$ NKT-like cells compared with healthy controls. Loss of CD28 was associated with an increase in percentage of NKT-like cells producing IFNγ and TNFα and importantly, with a loss of GCR (8) (**Figure 2C**). A significant loss of GCR in $CD8^+CD28^{null}$ NKT-like cells was noted in both COPD patients and controls compared with $CD8^+CD28^+$ NKT-like cells (mean ± SEM: 9 ± 4% $CD8^+GCR^+CD28^{null}$ NKT-like cells vs. 39 ± 7% $CD8^+GCR^+CD28^+$ NKT-like cells in COPD). There was a significant correlation between GCR expression and IFNγ and TNFα production by $CD8^+$ NKT-like cells. Taken together, these data show a loss of GCR in senescent $CD8^+CD28^{null}$ NKT-like cells and suggest that alternate treatment options to glucocorticoids are required to suppress pro-inflammatory cytokine production in patients with COPD.

Decreased Histone Deacetylase (HDAC)2 in $CD8^+CD28^{null}$ NKT-Like Cells in COPD

Histone acetyltransferases and HDAC are enzymes that upregulate and downregulate pro-inflammatory gene transcription, respectively. HDAC2 is required by corticosteroids to switch off activated inflammatory genes and is reduced in lung macrophages in COPD (10). A recent study showed that HDAC2 expression was suppressed in pro-inflammatory $CD8^+CD28^{null}$ NKT-like cells in patients with COPD (19) and negatively correlated with the percentage of $CD8^+CD28^{null}$ NKT-like cells producing IFNγ or TNFα in all subjects (e.g., COPD: $R = -0.789$, $p < 0.001$ for $CD8^+CD28^{null}$ NKT-like cells producing IFNγ) (**Figure 2D**). Theophylline is an activator of HDAC and enhances the anti-inflammatory effects of corticosteroids in alveolar macrophages in COPD patients (20). Addition of theophylline has recently been shown to increase the anti-inflammatory effects of steroids in senescent lymphocytes from COPD patients (18). Addition of low-dose theophylline (5 mg/l) induced a synergistic upregulation of HDAC2 in $CD8^+CD28^{null}$ NKT-like cells in the presence of 1μM pred and 2.5 ng/ml CsA (**Figure 2E**). This was associated with a decrease in pro-inflammatory cytokine production by these cells. These findings suggest this form of therapy may enhance the anti-inflammatory effects of steroids and thus reduce inflammation caused by these cells in COPD.

Decreased Heat Shock Protein (Hsp)90 in $CD8^+CD28^{null}$ NKT-Like Cells in COPD

Glucocorticoid receptor must be bound to molecular chaperones Hsp70 and Hsp90 to acquire a high-affinity steroid binding

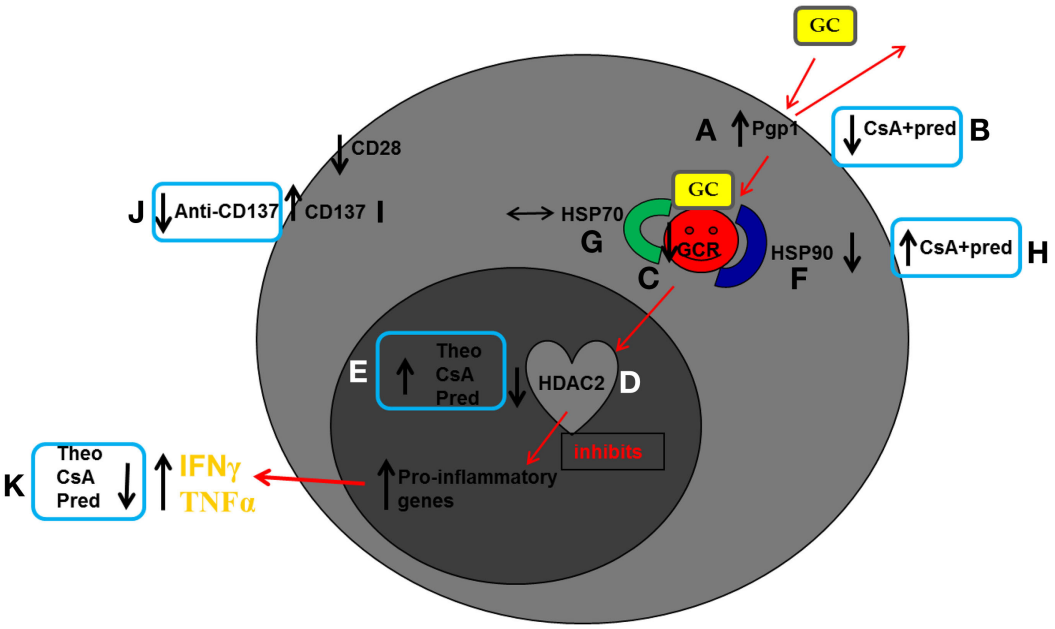

FIGURE 2 | Schematic diagram summarizing reported findings in peripheral blood CD8$^+$CD28^{null} natural killer T-like (NKT-like) cells in chronic obstructive pulmonary disease (COPD). Glucocorticoids enter cells by overcoming membrane drug efflux pump P-glycoprotein-1 (Pgp1) and binding to the glucocorticoid receptor (GCR) in the cytoplasm. GCR must be bound to the molecular chaperones heat shock protein (Hsp)70 and Hsp90 to acquire a high-affinity steroid binding conformation, and traffic to the nucleus where engagement of histone deacetylases (HDACs), particularly HDAC2, results in reduction of pro-inflammatory gene activation. In COPD compared with age-matched healthy control subjects: **(A)** Pgp1$^+$ NKT-like cells are increased in COPD, reducing intracellular levels of GC. Expression of GCR **(C)**, Hsp90 **(F)**, and HDAC2 **(D)** are decreased in CD8$^+$CD28^{null} NKT-like cells (no change in Hsp70) **(G)** reducing steroid effectiveness. **(I)** The percentage of steroid resistant CD8$^+$CD28^{null}CD137$^+$ NKT-like cells is increased. Possible therapeutic targeting to overcome steroid resistance CD8$^+$CD28^{null} NKT-like cells in COPD: **(B)** Pgp1 is synergistically decreased in the presence of 2.5 ng/ml cyclosporine A (CsA) and 1μM prednisolone (pred). **(H)** Hsp90 expression is increased in the presence of 2.5 ng/ml CsA and 1μM pred. **(E)** HDAC2 expression is increased in the presence of 5 mg/ml theophylline, 2.5 ng/ml CsA, and 1μM pred. **(J)** Blocking CD137 expression with anti-CD137 antibody. **(K)** This targeting results in decreased IFNγ and TNFα pro-inflammatory cytokine expression.

conformation and traffic to the nucleus (21). A recent study examined expression of Hsp70/90 in CD8$^+$CD28^{null} NKT-like cells from the peripheral blood of patients with COPD (22). Loss of expression of Hsp90 and GCR from the CD8$^+$CD28^{null} NKT-like cells in COPD was noted (**Figure 2F**), whereas expression of Hsp70 was unchanged (**Figure 2G**). The loss of Hsp90 was shown to correlate with the cytotoxic/pro-inflammatory potential of these cells and importantly, degree of airflow limitation in patients with COPD. The immunosuppressant, CsA, binds to the GCR–Hsp90 complex, but not Hsp70 (23), and was shown to upregulate Hsp90 with an associated decrease in pro-inflammatory cytokine production by CD8$^+$CD28^{null} NKT-like cells when combined with 1μM pred (**Figure 2H**). The concentration of CsA (2.5 ng/ml) used in these *in vitro* experiments was 50 times less than that used for patients following lung transplant to prevent graft rejection. Hence, these low concentrations are not likely to be associated with any of the side effects reported with higher doses of this drug.

INHIBITING CD137 EXPRESSION IN CD8$^+$CD28^{null} NKT-LIKE CELLS IN COPD

The loss of CD28 on CD8$^+$CD28^{null} NKT-like cells from COPD subjects has been reported to be associated with an upregulation of the "alternate" co-stimulatory molecule CD137 (4-1BB) (24) (**Figure 2I**). Targeting CD137 has been shown to be effective in treatment of rheumatoid arthritis and may thus be effective in other diseases associated with increased expression of this co-stimulatory molecule, including COPD (25). *In vitro* studies showed that blocking CD137 with an anti-CD137 antibody following PHA stimulation of PBMC from COPD patients resulted in a decrease in the percentage of CD8$^+$CD28^{null} NKT-like cells producing IFNγ, TNFα, and granzyme b production (26) compared with CD8$^+$CD28$^+$ NKT-like cells (**Figure 2J**), whereas stimulatory CD137 antibody increased production of these molecules. This indicates that targeting CD137 with anti-CD137 antibody may have novel therapeutic options for reducing inflammation in patients with COPD.

DOES OXIDATIVE STRESS PLAY A ROLE IN STEROID RESISTANCE IN NKT-LIKE CELLS?

There is increasing evidence that oxidative stress is important in the pathogenesis of COPD (27, 28). Oxidative stress occurs due to an increase of reactive oxygen species (ROS) causing damage to lipids, proteins, and DNA. Increased burden of oxidants from

cigarette smoke and air pollutants and from ROS and reactive nitrogen species (RNS) released from inflammatory neutrophils, eosinophils, macrophages, and epithelial cells occurs in the lungs of COPD patients (27–29). The aging process is associated with a decrease in the antioxidant defense mechanisms in the lung resulting in increased ROS and RNS (30). Although there is a causal link between ROS, COPD, and aging in cellular senescence in many cells in the lung, sensitivity of individual lymphocyte subsets to oxidative stress and how this process affects disease progression remains largely unknown (30). While one study showed an association between ROS and cellular senescence in lymphocytes, some markers of oxidative stress were decreased (31). Increasing concentrations of ROS has been shown to suppress Th1 cells and increase Th2 cells, findings at odds with ours and many others in patients with COPD (30). Furthermore, it has been shown that neutrophils in the inflamed lung produce large amounts of ROS, which suppress T cells, while macrophages secrete cysteine and thioredoxin, which increase oxidation resistance of T cells (32). Although oxidative stress has been shown to inhibit expression of GCRs in total blood leukocytes, the effect on T and NKT-like cells was not determined (33). It is clear further research is needed specifically on the effect of ROS on T cell and NKT-like cell biology (32).

FUTURE THERAPY FOR COPD

Lymphocyte senescence and glucocorticoid resistance have been described in several other inflammatory conditions such as cardiovascular disease (34), autoimmune disease (35), arthritis (36), IBD (37) associated with aging (38), and aging with associated inflammation in COPD (39). Some of these conditions are associated with respiratory muscle dysfunction resulting in further increases in ROS and oxidative stress (40). $CD28^{null}$ T cells have been reported in patients with asthma (41), another inflammatory lung disease also associated with increased ROS and oxidative stress (42). Interestingly, several of these inflammatory diseases are also comorbid conditions associated with COPD (10) and therefore may also be associated with increased cytotoxic/pro-inflammatory $CD8^+CD28^{null}$ NKT-like cells. Hence, targeting the pro-inflammatory nature of these cells by decreasing the expression of Pgp1 and/or CD137 and increasing the expression of GCR, HDAC2, and Hsp90 by $CD8^+CD28^{null}$ NKT-like cells may reduce inflammation (**Figure 2K**) associated with a range of steroid resistant diseases including COPD and comorbid conditions associated with COPD. Furthermore, targeting these cytotoxic/pro-inflammatory cells at early onset of COPD may prevent the inevitable spiral of worsening lung function, and associated comorbidity of this progressive debilitating disease, and reduce the associated massive health-care costs (43).

AUTHOR CONTRIBUTIONS

GH and SH organized, wrote, and edited the manuscript. Figures were drawn by GH and edited by SH.

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Human CD8+ T Cells in Asthma: Possible Pathways and Roles for NK-Like Subtypes

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Asthma affects approximately 300 million people worldwide and is the most common chronic lung disease, which usually is associated with bronchial inflammation. Most research has focused upon the role of CD4+ T cells, and relatively few studies have addressed the phenotypic and functional roles of CD8+ T cell types and subtypes. Human NK-like CD8+ T cells may involve cells that have been described as CD8+CD28−, CD8+CD28−CD57+, CD8+CD27−, or CD8+ effector memory (TEM) cells, among other. However, most of the data that are available regarding these various cell types were obtained in murine models did not thoroughly characterize these cells with phenotypically or functionally or did not involve asthma-related settings. Nevertheless, one may conceptualize three principal roles for human NK-like CD8+ T cells in asthma: disease-promoting, regulatory, and/or tissue repair. Although evidence for some of these roles is scarce, it is possible to extrapolate some data from overlapping or related CD8+ T cell phenotypes, with caution. Clearly, further research is warranted, namely in terms of thorough functional and phenotypic characterization of human NK-like CD8+ T cells in human asthma of varying severity.

Keywords: asthma, CD8, CD28, CD27, CD57, human, NK-like, T cells

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GENERAL ASPECTS ABOUT ASTHMA

Asthma affects approximately 300 million people worldwide and is the most common chronic lung disease (1). It is defined by a history of respiratory symptoms such as wheeze, shortness of breath, chest tightness, and cough that vary over time and in intensity, together with variable expiratory airflow limitation (2). In addition, it is a heterogeneous disease, usually characterized by chronic airway inflammation (2).

GENERAL ASPECTS ABOUT THE ROLE OF CD8+ T CELLS IN HUMAN ASTHMA

Although there are different clinical and cellular phenotypes in asthma, most research in terms of the role of T lymphocytes in this disease has been focused upon CD4+ T cells in the context of chronic airway inflammation. These CD4+ T cells produce various cytokines, namely IL-4, IL-9, and IL-13, that may contribute toward the underlying inflammation in asthma. In contrast, studies focusing on the role of CD8+ T cells in asthma have been comparatively scarce. Furthermore, most of the data

were obtained in murine models, and contradictory results have been produced by various groups, in terms of a possible role for these cells. In this context, possible pro-inflammatory, disease-inducing roles versus protective or "regulatory" roles have been suggested by different studies, as recently reviewed by Baraldo et al. (3). It should be borne in mind that, in the various existing studies, CD8+ T cells were retrieved and characterized using different methodological approaches in terms of patient groups or murine models, biological samples, and study methods. These aspects may account for most of the discrepancies observed, which have indeed been clustering around a non-relevant role (4), a protective role (5), or a disease-favoring role, including association with poorer lung function (6–10) and possibly involving a significant direct and indirect contribution toward a Th2-high bronchial inflammation (11, 12). Nevertheless, it should also considered that different conditions of the local milieu in the target organ, possibly involving exposure to different cytokines and/or antigen-producing cells may also explain discrepant results across existing studies. In addition, some experiments in murine models have also shown that the timing of the experimental setup with CD8+ T cells may also be very relevant in terms of the results observed. In this context, CD8+ T cells that produce high levels of IFN-gamma (Tc1 cells) have been shown to be associated with an attenuation of pulmonary allergic inflammation in rodent models. However, their role appears to depend upon their temporal relationship with the progression of allergic sensitization. In fact, depletion of CD8+ T cells prior to systemic OVA sensitization, either by blocking antibody directed against CD8+ or by using knockout mice, tends to be associated with attenuated allergic inflammation and bronchial hyperresponsiveness (BHR) in response to antigen challenge. However, administration of CD8+ blocking antibody after the initial allergen sensitization procedure results in further increase in BHR and eosinophilia (13).

Finally, it should be emphasized that many studies focusing on CD8+ cells in asthma have utilized different approaches to phenotypically characterizing these cells. In fact, in some cases, it is not clear whether the observed cells are classical CD8+ T cells, regulatory T cells, or NK-like CD8+ T cells.

GENERAL ASPECTS ABOUT THE ROLE OF HUMAN NK-LIKE CD8+ T CELLS IN ASTHMA

As far as we know, there are no published studies on human NK-like CD8+ T cells, as usually defined, in asthma. Human NK-like CD8+ T cells most likely comprise a whole array of cells with different phenotypes and functions, and further research is warranted in order to thoroughly clarify their ontogeny, patterns of differentiation, different phenotypes, and functions (**Figure 1**). In humans, NK-like T cells are a subset of CD8+ T cells that express prototypical NK cell markers, such as CD56, CD161, CD16, CD94, and CD57, with such expression increasing with aging. In most cases, these CD8+ T cells do not express CD28 (14–16). For the sake of clarity, we will restrict our concept of human NK-like CD8+ T cells to CD8+ T lymphocytes that have been termed concurrently or independently CD28−CD8+ T cells, CD27−CD8+ T cells, CD28−CD57+CD8+ T cells, CD8+ effector memory T cells (T_{EM}). A few data exist regarding these possible types of NK-like CD8+ T cells in humans, particularly in the setting of respiratory diseases such as asthma. In addition, given the fact that differences are apparent between these cells in mice and humans, we will only resort to information from murine models where strictly necessary.

CD8+CD28− T cells can be regarded as one of the possible subtypes of NK-like CD8+ T cells. There is a progressive oligoclonal accumulation of CD8+CD28− (and CD57+) T cells with natural aging, possibly due to multiple rounds of immune responses to antigenic exposures (17). In fact, chronic, persistent immune stimulation has been shown to be associated with reciprocal loss of CD28 expression and gain of CD57 expression on human, but not murine, CD8+ T cells (18, 19). Thus, most human CD8+CD28− T cells are CD57+, have shortened telomeres, represent late-differentiated T cells, and have been shown to express high levels of granzymes and perforin (20).

Various other phenotypic markers have been studied on CD8+CD28− T cells, namely CD27, and these phenotypes have been associated with different subtypes of memory T cells. It is generally accepted that human classical memory cells that have differentiated into an effector-like type (T_{EM}) tend to be CD27− and CD28−, express perforin and granzymes, have moderate cytotoxic capacity but are capable of producing high levels of cytokines, namely IFN-gamma and TNF-alpha (21–23).

Can Human NK-Like CD8+ T Cells Have a Pro-Inflammatory Role in Asthma?

In a study involving postmortem peribronchial region samples, the percentage of CD8+CD25+ T cells and perforin expression was higher in patients who had died from asthma (AD) than in asthmatic patients who had died of unrelated causes or in individuals who had died without a history of lung diseases (control groups) (6). In addition, the IFN-gamma/IL-4 ratio was lower in the AD than in the control groups.

Another study, involving asthmatic patients who were followed up for 14 years showed that the decline in lung function (FEV_1), although mild, was clearly correlated with the number of CD8+ T-cells in airway biopsies, not just at baseline but also on follow-up (5). Curiously, a recent study in asthmatic patients demonstrated that the degree of airflow obstruction (FEV_1 and $FEF_{25–75}$) was correlated not with the total number of CD8+ T cells but rather with the number of CD8+ T-cells in the bronchoalveolar lavage fluid which expressed the high-affinity receptor for leukotriene B4 (BLT1) and produced IL-13 (10). Furthermore, the numbers of these cells also correlated with serum IgE levels and with the airway basement membrane thickness. However, CD8+ T cells were not further phenotyped in these studies, and we cannot know whether they also contained a subpopulation of NK-like CD8+ T cells.

Other studies have shown that CD8+CD28− (CD57+) T cells may be associated with inflammation in asthma, particularly in more severe cases. In this regard, the induced sputum of asthmatic patients was shown to contain more CD8+CD28− (CD57+)

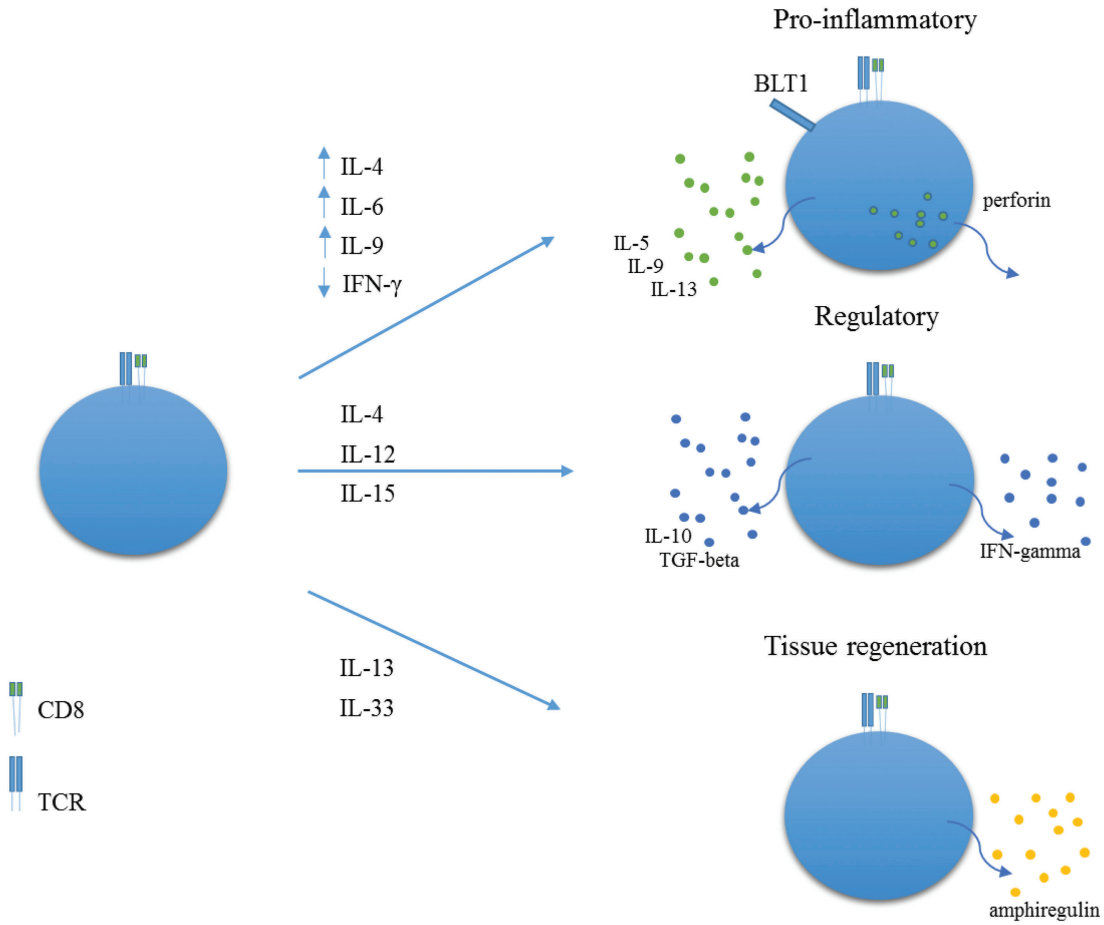

FIGURE 1 | Proposed roles for NK-like CD8+ T cells. In environments rich in IL-4, IL-6, IL-9, and low IFN-gamma, NK-like CD8+ T cells may acquire a pro-inflammatory role with expression of BLT1 and production of perforin, IL-5, IL-9, and IL-13. In environments rich in IL-4 and IL-12 or IL-15, NK-like CD8+ T cells may differentiate into regulatory T cells, with production of IFN-gamma, TGF-beta, and IL-10. In pro-inflammatory contexts, rich in IL-18 and IL-33, NK-like CD8+ T cells may produce amphiregulin, thereby promoting tissue regeneration.

T cells than CD8+CD28+ T cells, in contrast to what was observed in healthy controls. Furthermore, these CD8+CD28− T cells were also shown to be more abundant and to contain lower levels of IFN-gamma in severe asthmatics than in mild asthmatics and age-matched healthy controls. Furthermore, CD8+CD28− (CD57+) T cells from severe asthmatics expressed high levels of intracytoplasmic perforin and demonstrated a clearly more potent cytotoxic activity than in CD8+CD28− T cells from healthy controls and mild asthmatics (24). To what extent this increased cytotoxic activity is relevant to inflammation in asthma still needs to be ascertained.

Clearly, further studies are needed, particularly in terms of the cytokines produced by these CD8+CD28− T cells in asthma. A lower production of IFN-gamma in asthma may be relevant in that it may be less effective at counterbalancing Th2-associated cytokines. In this regard, it should be stressed that, just like CD4+ T cells, CD8+ T cells can also be subdivided into T1-type (Tc1) and T2-types (Tc2), on the basis of the cytokines they produce (25, 26). Thus, some CD8+ T cells produce high levels of IL-4 and/or IL-13, possibly under the influence of IL-4 produced by Th2-type CD4+ T cells, as was shown in a murine model (27). The relevance of IL-4 produced by these CD8+ T cells is not clear, but it may contribute toward tissue remodeling (28). This is even more relevant since IL-4 production has been demonstrated in peripheral blood CD8+ T cells from patients with atopic asthma (29). More importantly, IL-13 may also significantly contribute toward bronchial inflammation, airway hyperresponsiveness, mucus hypersecretion, and tissue remodeling as has been shown in murine models and models of human bronchial epithelium (28, 30, 31).

In addition, a subset of effector memory (T_{EM}) CD8+ T cells with high levels of IL-6Ra expression in human peripheral blood was reported. IL-6Ralpha^{high} EM CD8+ T cells actively proliferated, survived, and produced high levels of IL-5 and IL-13. Also, patients with asthma had an increased frequency of IL-6Ralph^{high} CD8+ T cells (T_{EM}) in peripheral blood compared with healthy control subjects. It is possible that these cells may serve as a pool, which expands with immune stimulation (32).

Finally, CD8+ T cells may also produce other cytokines that are very relevant to the pathophysiology of bronchial asthma, such as IL-9 (Tc9 cells) and IL-17 (Tc17 cells), which may have a role even in Th2-low settings (11, 33). In fact, a recent study showed that the numbers of peripheral blood Tc2 and Tc17 cells were increased in asthmatic versus non-asthmatic individuals (33). Again, production of these cytokines should be analyzed in human NK-like CD8+ T cells.

Can Human NK-Like CD8+ T Cells Have a Regulatory Function in Asthma?

In the case of CD8+ T cells, two of the possible ways these cells might have a regulatory function in asthma would be *via* high production of IFN-gamma and/or *via* mechanisms generally associated with "regulatory" T cells and involving the production of IL-10 or TGF-beta or direct cell-cell contact-associated suppression.

Increased expression of IFN-gamma producing CD8+ T cells has been demonstrated in subjects with asthma (12, 34), although a decreased expression of IFN-gamma in CD8+ T cells in atopic asthmatic patients has also been described (35) and CD8+ T cells from atopic asthmatic subjects have been shown to contain more IL-4 than those from non-atopic donors (29). In fact, memory CD8+ T cells can be activated in the presence or absence of specific antigen expressed by dendritic cells, in association with the pro-inflammatory cytokines IL-15 and IL-18, to produce IFN-gamma that leads to the suppression of the underlying Th2-driven allergic airway inflammation (36).

In fact, in the presence of IL-4 and IL-12, murine CD8+ T cells have been shown to become CD39+ Foxp3-negative "regulatory" T cells that demonstrate suppressive activity *via* production of IL-10 and contact-dependent mechanisms (5). Furthermore, memory CD8+ T cells present in the airways of mice after an influenza infection have been shown to suppress the development of subsequent Th2-driven allergic inflammation in an IFN-gamma dependent way (37). In addition, the adoptive transfer of IFN-gamma-producing CD8+ T cells directly into the airways suppressed the allergic response in pre-sensitized mice (36). However, to what extent these CD8+ Tregs are CD28− has not been described.

It is thought that naïve CD8+CD25+ cells can differentiate into CD8+ Tregs in the presence of antigen and the relevant cytokines (38). As an example, human CD8+ Treg can be generated in the presence of IL-4 and IL-12; these cells are CD25+Foxp3+ and are capable of secreting IL-10, TNF-alpha, IFN-gamma as well as granzymes (39). Furthermore, these cells have been shown to block the activation of naïve or effector T cells, to suppress IgG/IgE antibody responses (39), IL-4 expression, and the proliferation of CD4+ T cells (40). However, most of these cells described in humans are CD28+ (39–41) and most likely do not involve NK-like CD8+ T cells. An alternative pathway in terms of CD8+ T cell differentiation toward Tregs may involve IL-15. In this context, human CD8+CD56− T cells, stimulated with IL-15, were shown to acquire the capacity to secrete IFN-gamma, IL-1beta, TGF-beta, and IL-10, suggesting a regulatory phenotype (42).

It should be stressed that a subset of human CD8+CD28− T suppressor cells, which were shown to act upon antigen-presenting cells, rendering them tolerogenic to CD4+ T cells were described in a model of mixed lymphocyte reaction (43). Phenotypic analyses of these CD8+CD28− T cells showed that they were CD3+, $CD5^{high}$, $CD8^{high}$, CD27+, CD56−, CD62L+ (44) opening up the possibility of the existence of CD8+CD28− Tregs in humans.

In human asthma, flow cytometry analysis showed an increased percentage of CD8+CD28− T cells in peripheral blood of adult allergic asthmatics compared to controls (45). In addition, patients with severe asthma had a higher percentage of CD8+CD28− and CD8+CD28−TCRalpha/beta+$CD62L^{high}$ $FoxP3^{bright}$ T cells than the other groups after enrichment, suggesting that these cells might not be immunosuppressive or that their increased numbers in asthma might indicate a tissue damage-limiting function, as happens in the context of viral infections [reviewed by Josefowicz et al. (46)]. In contrast, the same group of researchers showed that the percentages of peripheral blood CD8+CD25+$FoxP3^{bright}$ T cells of patients with severe asthma or mild to moderate asthma were markedly lower than those of non-asthmatic controls (47). Curiously, the percentages of CD8+CD25+$FoxP3^{bright}$ T cells correlated with mean peak expiratory flow (PEF%) values in these asthmatic patients (47). Although this study did not analyze whether these CD8+ Tregs were CD28− (and/or CD57+), joint analysis of the results from the studies by these researchers may suggest that the CD8+CD28− described in their reports are not true immunosuppressive Tregs, which is in line with results from various other groups that have described CD8+CD28− T cells as essentially cytotoxic and not immunosuppressive (24, 48–51). Furthermore, other authors have also shown that human CD8+CD57+ T cells are mostly cytotoxic, at least those that are present in the context of autoimmune diseases (52–56).

Nevertheless, the picture is not clear at all, since a clear immunosuppressive activity carried out by CD8+CD57+ T cells (57–59) as well as by CD8+CD28− T cells (44, 60–63) has been described, but in the context of tissue transplantation and autoimmune diseases in humans. To what extent this may apply to allergy and asthma needs to be determined.

Thus, in order to clarify this issue of human NK-like (CD8+CD28−) T cells having or not "regulatory" properties in human asthma, further studies are needed, involving a thorough phenotypic and functional characterization of both peripheral blood and bronchial CD8+CD28−CD57+ as well as CD8+CD28−CD57− T cells in patients with bronchial asthma of different degrees of severity as well as in non-asthmatic controls.

Can Human NK-Like CD8+ T Cells Have a Tissue-Regenerating Function in Asthma?

Amphiregulin is an epidermal growth factor ligand, which apparently promotes tissue repair under inflammatory conditions [reviewed by Berasain and Avila (64)]. Studies in a murine model have shown that, through the production of amphiregulin, Treg cells have a direct role in lung tissue repair and maintenance during viral infection that is independent of their suppressive activity and is induced by different stimuli (65). In this context, the pro-inflammatory cytokine IL-18 and the alarmin IL-33,

which are upregulated in the context of inflammation and tissue damage, induced *in vitro* amphiregulin production by Treg, independently of TCR stimulation. However, this study did not fully clarify which type of Tregs was involved, although mostly CD4+ Tregs were studied.

Amphiregulin is also produced by human T cells, as shown in a study in which signaling through the TCR induced amphiregulin expression by most or all human T cell subsets in peripheral blood, including naive and memory CD4+ and CD8+ T cells, Th1 and Th2 *in vitro* T cell lines, and subsets of memory CD4+ T cells (66). In these different T cell types, amphiregulin synthesis was regulated essentially by acute signals, which may be appropriate for tissue repair. Importantly, amphiregulin-producing CD4+Foxp3+ Tregs have been described in damaged tissues, namely muscles in murine models (67). Furthermore, the specialized proresolving mediator maresin-1 has been shown to reduce asthma-associated bronchial inflammation in a murine model, and this was associated with an increased expression of amphiregulin and *de novo* generation of CD4+ Tregs (68). It is, thus, possible that damaged bronchial tissue in cases of severe asthma may be associated with the local accumulation of amphiregulin-producing Treg. However, amphiregulin expression has not been studied in human NK-like, CD8+CD28− T cells. In addition, amphiregulin expression in asthma must be interpreted with caution, since elevated levels of amphiregulin have been detected in induced sputum in asthmatic children, where its levels correlated with the numbers of sputum eosinophils and sputum eosinophil cationic protein (69). Furthermore, there was a significant negative correlation between sputum amphiregulin and lung function (FEV_1). Finally, another study in asthmatic children showed that levels of amphiregulin in the sputum were increased in disease exacerbations (47). In addition, in this study, amphiregulin was also shown to induce proliferation of normal human bronchial epithelial cells, which may be associated with tissue remodeling in asthma.

CONCLUSION

Independently of other reasons, seemingly contradictory findings regarding human CD8+ T cells, namely NK-like CD8+ T cells may be due to different populations/subpopulations involved in the different experimental setups used. In addition, a clearer definition of the CD8+ subsets both in phenotypic and functional terms would allow more useful comparisons between studies both in animal models (rodent) and in human pathological contexts.

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Activation of Blood CD3$^+$CD56$^+$CD8$^+$ T Cells during Pregnancy and Multiple Sclerosis

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A striking common feature of most autoimmune diseases is their female predominance, with at least twice as common among women than men in relapsing–remitting multiple sclerosis (MS), the prevailing MS clinical form with onset at childbearing age. This fact, together with the protective effect on disease activity during pregnancy, when there are many biological changes including high levels of estrogens and progesterone, puts sex hormones under the spotlight. The role of natural killer (NK) and NKT cells in MS disease beginning and course is still to be elucidated. The uterine NK (uNK) cells are the most predominant immune population in early pregnancy, and the number and function of uNK cells infiltrating the endometrium are sex-hormones' dependent. However, there is controversy on the role of estrogen or progesterone on circulating NK (CD56^{dim} and CD56^{bright}) and NKT cells' subsets. Here, we show a significantly increased activation of CD3$^+$CD56$^+$CD8$^+$ cells in pregnant MS women (MSP) compared with non-pregnant MS women (NPMS) ($p < 0.001$) and even with respect to healthy pregnant women (HP, $p < 0.001$), remaining increased even after delivery. The dynamics of expression of early activation marker CD69 on CD3$^+$CD56$^+$CD8$^+$ cells showed a progressive statistically significant increase along the gestation trimesters (T) and at postpartum (PP) with respect to NPMS (1T: $p = 0.018$; 2T: $p = 0.004$; 3T: $p < 0.001$; PP: $p = 0.001$). In addition, early activation expression of CD69 on CD3$^+$CD56$^+$CD8$^+$ cells was higher in MSP than HP in the first two trimesters of gestation ($p = 0.004$ and $p = 0.015$, respectively). NPMS showed significantly increased cytotoxic/regulatory NK ratio compared with healthy controls ($p < 0.001$). On the other hand, gender studies showed no differences between MS women and men in NK and CD3$^+$CD56$^+$CD8$^+$ cells' subsets. Our findings may add on the understanding of the regulatory axis in MS during pregnancy. Further studies on specific CD8$^+$ NKT cells function and their role in pregnancy beneficial effects on MS are warranted to move forward more effective MS treatments.

Keywords: pregnancy, regulatory immune response, postpartum, NK cell, CD3$^+$CD56$^+$CD8$^+$ cells, multiple sclerosis

INTRODUCTION

Multiple sclerosis (MS) is a prototypic autoimmune inflammatory disorder of the central nervous system (CNS), in which both adaptive and innate immune systems are assumed to participate in demyelination and neurodegeneration (1). Epidemiological data indicate that relapsing–remitting-MS is more prevalent in females than in males (3–2:1) (2), while men tend to develop a more severe and chronic form of the disease. Several factors have been proposed to contribute to gender bias in susceptibility to MS. The fact that MS is typically diagnosed in the fertile age turns to sex hormones, an important focus of study. For female patients with MS, pregnancy is one of the strongest known modulators of disease activity (3, 4), largely attributed to elevated levels of circulating sex hormones, such as estrogen or progesterone.

Natural killer (NK) cells are innate immune cells with a major role in eliminating virus-infected and tumor cells. Current evidences on the role of peripheral NK cells in exacerbating or dampening autoimmunity responses in MS are controversial (5). Peripheral NK cells' activity has been reported to be reduced in the setting of MS (6–8), although its role in the pathophysiology of the disease is largely unknown. On the other hand, the uterine natural killer (uNK) cells are the most predominant immune population at the decidua in early pregnancy and disclose a regulatory phenotype, while its origin remains to be determined. However, effects of sex hormones on the immune system, and more specifically on peripheral NK and NKT cells, have not been clearly elucidated.

Two major functionally distinct subsets of NK cells have been described based on the relative expression of the markers CD16 and CD56 (9). Typically, the majority (approximately 90%) of circulating NK cells have low expression of CD56 and high levels of CD16 and perforin, and display potent cytolytic activity (cytCD56^{dim}). This subset can not only spontaneously lyse targeted tumor cells but also induce rapid inflammatory responses by releasing significant amounts of chemokines and proinflammatory cytokines when their activating receptors are engaged. By contrast, NK cells expressing high levels of CD56 and low CD16 are more abundant in secondary lymphoid tissues and tonsils (10, 11), and comprise nearly 70% of human decidual lymphocytes (9). Following monokine stimulation, CD56^{bright}CD16^{-} proliferate and produce immunoregulatory cytokines, including IFN-γ, TNF-α, and GM-CSF, and there is a general consensus in the literature that ascribe them a protective role in the neuroimmunological context in MS (regCD56^{bright}) (12).

NKT cells comprise a small subset of lymphocytes that possesses characteristics of both NK cells and conventional T cells, being able to release prototypical Th1 and Th2 cytokines after TCR ligation (13), and to induce perforin-, Fas-, and TNF-related cytotoxicity (14). Thus, these cells can have either protective or deleterious effects by promoting either inflammation or immune tolerance. Several studies have highlighted their regulatory role in autoimmune diseases, such as MS, type I diabetes mellitus, primary biliary cirrhosis, systemic lupus erythematosus, rheumatoid arthritis, psoriasis, and atherosclerosis, among others (15). NKT cells recognize lipid or glycolipid antigens presented by the MHC class I-related protein CD1d and exert their multiple functions, including antibacterial and antiviral immune responses, tumor-related immunosurveillance or immunosuppression, and inhibition or promotion of the development of autoimmune diseases (16). Few studies have revealed alterations in the numbers (17, 18) and functions of NKT cells in MS patients, despite MS is an organ-specific disease in which myelin lipids are a major target [reviewed in Ref. (19)]. The fact that specific NKT cells prevalence and function is restored in MS patients in remission after IFN-β treatment (12); that oral corticosteroids induce a Th2 bias in the cytokine profile of these cells (20); and that 1,25(OH)D3 vitamin induce protection from EAE in mice dependent of NKT cell-derived IL-4 (21) suggests that NKT cells might exert immunoregulatory more than detrimental effects in MS. Depletion of iNKT in mice show a more severe EAE course (22). On the contrary, expanding iNKT protects from EAE by suppressing Th1 and Th17 responses (23, 24). Type II NKT cells exert also protective effects on EAE models (25). Further, activation of NKT cells with synthetic lipid antigens protects mice against the development of MS-like disease [reviewed in Ref. (26)]. Very little is known about the CD8^{+}NKT role in MS. Interactions with other immunoregulatory cell types, such as regulatory T cells and immunosuppressive myeloid cells, might exert immunoregulatory effects in MS by producing Th2 cytokines [reviewed in Ref. (20)]. Further, CD8^{+}NKT cells can function as antigen-specific suppressive cells to regulate the immune response through killing antigen-bearing DCs (27) and efficiently suppress the proliferation and expansion of activated T cells (28).

In this study, we sought to determine, first, the distribution of NK and NKT-like cells subsets according to sex and their involvement in the disease and pregnancy. Second and given the role of uNK cells in maternal tolerance during pregnancy, we explored the distribution of the NK and NKT-like cell subsets during normal and MS pregnancy, to ascertain whether they exert a role during pregnancy. To address this goal, we evaluated the proportion and activation status (measured by CD69 expression) of circulating NK subsets (regCD56^{bright} and cytCD56^{dim}) and CD3^{+}CD56^{+}CD8^{+} cells in both men and women from healthy subjects, MS patients, and pregnant women. Finally, we measured estrogens and progesterone levels in the luteal and follicular phases of the menstrual cycle of healthy and MS women to assess the effects of the menstrual cycle and gender on NK activation.

PATIENTS AND METHODS

Subjects

A total of 124 subjects were studied. Among them, 70 MS patients, 30 non-pregnant women (mean age 39.0 years, range 28–51), 10 men (mean age 36.5 years, range 30–43), and 30 pregnant women (mean age 34 years, range 31–36) were consecutively recruited at the Unit of Multiple Sclerosis of the University General Hospital Gregorio Marañón of the Community of Madrid, Spain. All patients fulfilled definite MS diagnosis according to McDonald's criteria (1). These patients had not received any immunomodulatory or immunosuppressive therapy in the previous 3 months and had non-active disease at the time of sample collection.

A group of 54 age-matched healthy controls were recruited from volunteers at University General Hospital Gregorio Marañón during the same period: 32 women (mean age 28.1 years, range 21–39), 9 men (mean age 31.6 years, range 21–40), and 13 pregnant women (mean age 33 years, range 30–37). All 32 healthy women included in the study had regular menstrual cycles and were studied at days 1–3 and at day 14 (ovulation) of their menstrual cycle. None of HC and MS female had received treatment with glucocorticoids and contraceptive pills prior to the study inclusion. Clinical and demographic characteristics of individuals enrolled in the study are summarized in **Table 1**. The Ethics Committee of the institution approved the protocol, and all subjects provided their written informed consent.

TABLE 1 | Clinical and demographical characteristics of multiple sclerosis (MS) patients and healthy controls (HC) included in the study: pregnant MS women (MSP), non-pregnant MS women (NPMS), MS men, healthy pregnant women (HP), non-pregnant healthy control women (NPHC), and HC men.

	MSP	NPMS	MS men	HP	NPHC	HC men
No. of patients	30	30	10	13	32	9
Age (years)	34 (31–36)	39 (28–51)	36 (30–43)	33 (30–37)	28 (21–39)	31 (21–40)
EDSS	0 (1) (prior pregnancy)	1 (0–1.6)	1 (0.5–1.5)	NA	NA	NA

Data are expressed as median (interquartile range).
EDSS, Expanded Disability Status Scale.

NK and NKT Cell Subsets' Analysis

Lymphocyte subsets were analyzed using multiparametric flow-cytometry analysis (FacsCANTO, BD Biosciences, San José, CA, USA). Cells were directly stained with the following monoclonal antibodies according to the manufacturer recommendations: CD69-FITC (Mouse Anti-Human CD69, Clone L78; BD Biosciences), CD16-PE (Mouse Anti-Human CD16, Clone B73.1; BD Biosciences), CD3-PerCP (Mouse Anti-Human CD3ε, Clone SK7; BD Biosciences), CD56-APC (Mouse Anti-Human CD56, Clone NCAM16.2; BD Biosciences), and CD8-APC-Cy7 (Mouse Anti-Human CD8α, Clone SK1; BD Biosciences). IgG isotypic controls (BD Biosciences) were also tested to determine non-specific staining. Cells were incubated and protected from light at room temperature (RT) for 20 min. Afterward, cells were lysed (FACSTM-Lysing Solution; Becton Dickinson, San Jose, CA, USA), incubated again for 15 min in the dark, and then removed and washed with 2 mL phosphate-buffered saline. In the last step, a 6-color analysis was carried out using FACSCANTO flow cytometer (Becton Dickinson). Cell-Quest research (Becton Dickinson) and FlowJo (Tree Star, Ashland, OR, USA) software were used for the analysis. The gate was set for both FSC and SCC and included lymphocytes (**Figure 1**). A total of 20,000 events in the lymphocyte gate were acquired for each sample. After further gating on $CD3^-$ cells, the percentage of $CD3^-CD56^{bright}CD16^-$ and $CD3^-CD56^{dim}CD16^+$ NK cell subsets was determined. $CD3^+CD56^+CD8^+$ cells were analyzed in parallel on total lymphocytes. Although cell populations other than NKT cells might be included in the analysis, as minority subset of γδ-T cells, which

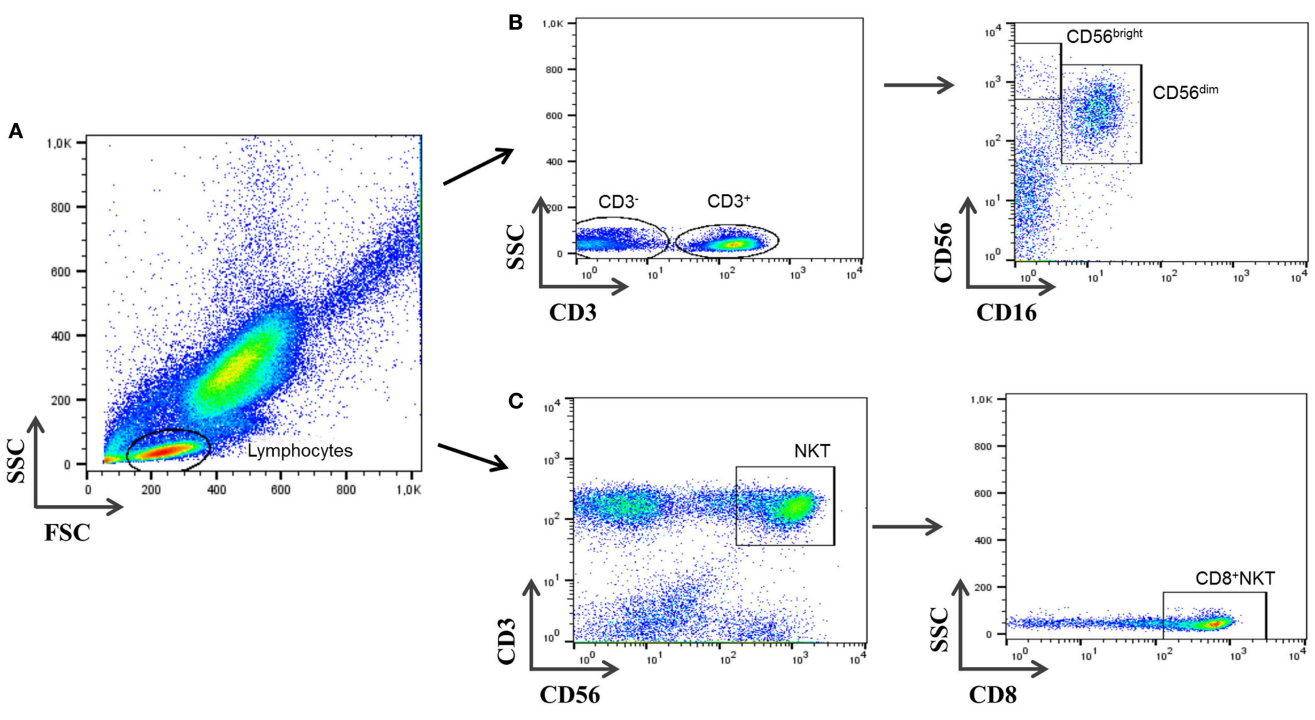

FIGURE 1 | Gating strategy for natural killer (NK) cells' subsets and $CD3^+CD56^+CD8^+$ cells. (A) Peripheral blood events were measured against forward and side scatter parameters, and total lymphocytes were selected. **(B)** Cells negative for CD3 were first selected and further displayed on a plot of CD16 vs. CD56 expression. Cells negative for CD16 and positive for CD56 are $CD3^-CD56^{bright}CD16^-$ NK cells and $CD3^-CD56^{dim}CD16^+$ NK cells. **(C)** $CD3^+CD56^+$ were analyzed gating on lymphocytes cells and selected from a plot of CD3 vs. CD56. $CD3^+CD56^+CD8^+$ cells were selected afterward.

represents less than 1% of total $CD3^+CD56^+CD8^+$ cells (data not shown), non-significant differences were observed in γδ-T cells among all groups studied. Differences in $CD3^+CD56^+CD8^+$ cells would be ascribed to the $CD8^+$NKT-like population. To avoid controversy and enhance accuracy of the nomenclature, we will use the term $CD3^+CD56^+CD8^+$ herein for this subset instead of "$CD8^+$ NKT-like cells." The $CD3^+CD56^+CD4^+$ T subpopulation was not considered for the analysis due to its very low percentage of the population studied. All NK and $CD3^+CD56^+CD8^+$ subsets are given as percentage of total lymphocytes.

Progesterone and Estrogen Hormonal Quantification

Blood samples were obtained around 8:30 a.m. Serum was separated in a refrigerated centrifuge and stored at −80°C until use. Serum progesterone and estrogen levels were determined in the luteal and follicular phases of the menstrual cycle of both healthy and MS women and men groups by a chemiluminescence assay (Immuno I, Bayer, Germany) following the manufacturer's instructions.

Statistical Analysis

Descriptive data are presented as median (interquartile range). When multiple groups with continuous outcomes were compared, the non-parametric Kruskal–Wallis rank sum test was used, followed by pairwise Mann–Whitney tests if the former indicated significant differences. Correlations were assessed using Spearman correlation (rs) coefficients. Data were analyzed with SPSS v.19 (Chicago, IL, USA) and GraphPad Prism software (CA, USA). A p-value less than 0.05 was considered as statistically significant.

RESULTS

NK and $CD3^+CD56^+CD8^+$ Cell Subsets' Changes within the Menstrual Cycle

As previously reported (29), no significant differences neither for the values of total NK cells nor for any of the NK cell subsets between the days 1–3 and 14 of the menstrual cycle of the control group of normal fertile women were found (data not shown). In parallel, no statistically significant differences in the percentage of $CD3^+CD56^+CD8^+$ cells during the two phases of menstrual cycle were observed. As a conclusion of this part, the study of NK and NKT cells could be performed at any time-point of the menstrual cycle. As expected, sex hormone levels significantly increased on day 14 of the menstrual cycle with respect to day 1–3 ($p < 0.001$): estrogens [39.6 (33.2–54.6) vs. 168.9 (100.0–584.7)] and progesterone [0.4 (0.3–0.6) vs. 3.3 (1.3–7.3)].

NK and $CD3^+CD56^+CD8^+$ Cell Subsets' Changes during Pregnancy

Healthy pregnant women (HP) showed an increased proportion of the regulatory $CD56^{bright}$ subpopulation [0.44 (0.3–0.73) vs. 0.33 (0.23–0.58); $p = 0.015$] compared to the non-pregnant healthy control women (NPHC) (**Figure 2**). No differences were observed in $CD3^+CD56^+CD8^+$ in HP and NPHC.

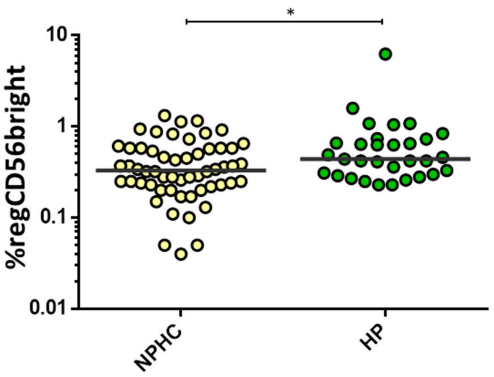

FIGURE 2 | Percentage of regCD56^{bright} in non-pregnant healthy control women (NPHC) ($n = 32$) and HP ($n = 13$). The bar represents the median value, and individual dots indicate single donor values. Mann–Whitney statistical test was used for calculation of the reported p-value. *$p = 0.015$ was considered to be statistically significant.

This pattern was not evident in MS pregnancies. Instead, MSP showed a marked early activation of the three NK/NKT populations studied. The most remarkable finding was the significantly increased $CD3^+CD56^+CD8^+$ activation compared with non-pregnant MS women (NPMS) ($p < 0.001$) and even with HP ($p < 0.001$) (**Figure 3A**). This increase remained statistically significant and progressed during all the trimesters (T) of gestation and at postpartum (PP) with respect to NPMS (1T: $p = 0.018$; 2T: $p = 0.004$; 3T: $p < 0.001$; PP: $p = 0.001$, respectively). Further, activation of $CD3^+CD56^+CD8^+$ cells was even higher in MSP than HP in the first two trimesters of gestation ($p = 0.004$; $p = 0.015$, respectively). Specifically, during the second and third trimesters of gestation, there was an increased activation of regCD56^{bright} NK in MS pregnancy compared to NPMS (2T: $p = 0.063$; 3T: $p = 0.037$) and even higher than HP during the second trimester ($p = 0.033$). Only at the third trimester, there was a slightly higher activation of the cytCD56^{dim} NK subpopulation with respect to NPMS and HP (MSP vs. NPMS: $p = 0.019$; MSP vs. HP: $p = 0.05$) (**Figure 3B**). No significant differences in the proportions of cytNK, regNK, and $CD3^+CD56^+CD8^+$ cells' subsets were observed between MSP and NPMS and between MSP and HP.

Regarding sex hormone levels, we did not observe differences during pregnancy between MSP and HP. However, a significant decreased estrogen levels in MSP with respect to HP was shown during the PP ($p = 0.04$). No correlation was showed among NK and NKT cell subsets and sex hormones.

Gender Effects on NK Cells

To evaluate gender effects, we analyzed global differences between non-pregnant women and men in both MS and healthy groups. Although estrogen levels have a significantly different distribution in women and men (healthy: $p < 0.001$; MS: $p = 0.002$), no gender differences were observed in the proportions of any NK and NKT populations studied in neither healthy nor MS patients.

There were, however, differences when comparing subsets between MS and the healthy group when considered by

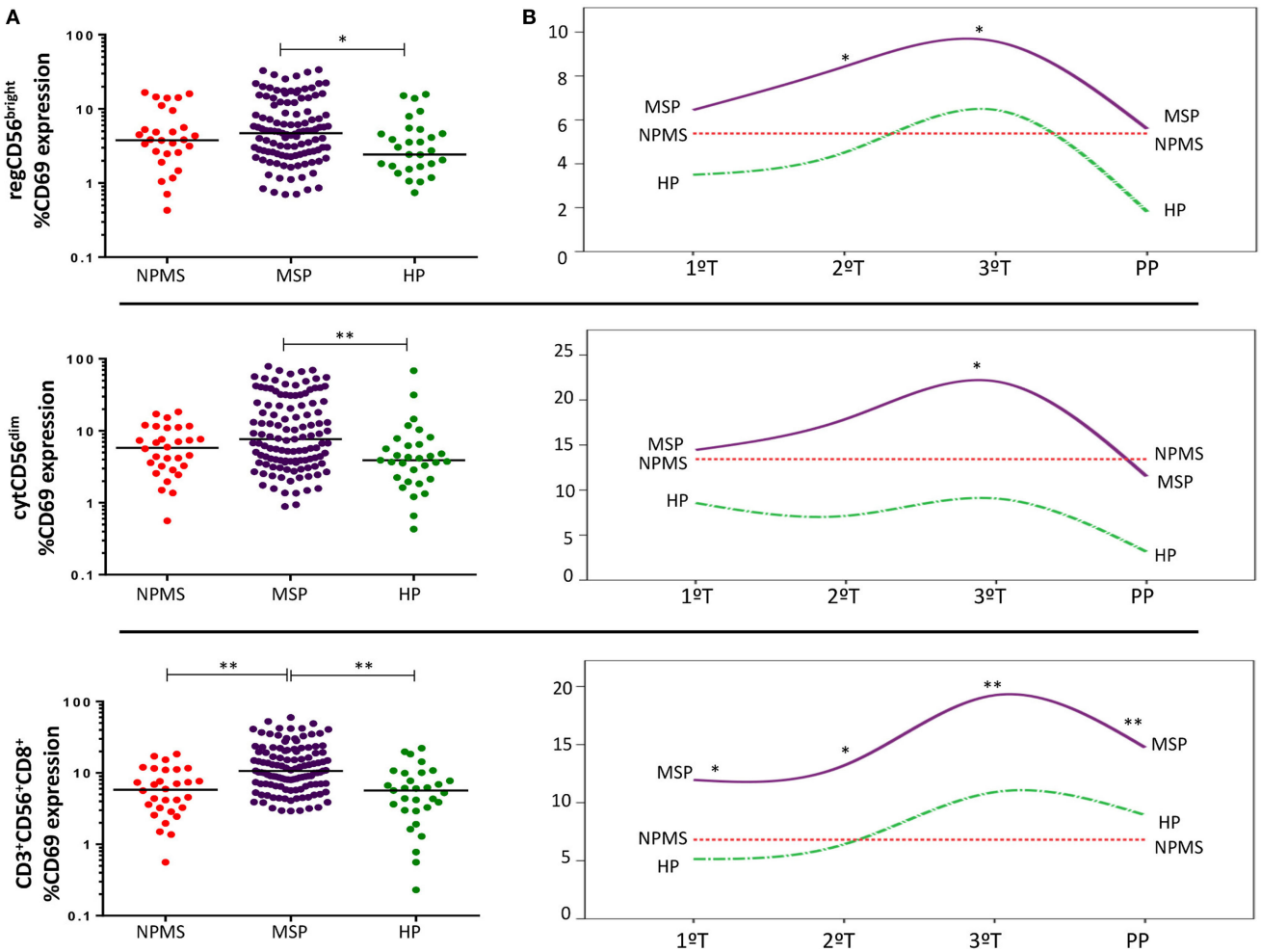

FIGURE 3 | **(A)** Early activation marker CD69 expression by peripheral blood regCD56^{bright} (CD3$^-$CD56$^+$CD16$^-$), cytCD56^{dim} (CD3$^-$CD56$^+$CD16$^+$), and CD3$^+$CD56$^+$CD8$^+$ cell subsets throughout pregnant multiple sclerosis (MS) women (MSP; $n = 30$), non-pregnant MS women (NPMS; $n = 30$), and healthy pregnant women (HP; $n = 13$). The bar represents the median value, and individual dots indicate single donor values. **(B)** CD69 expression longitudinally studied during each trimester of pregnancy (1T, 2T, 3T) and postpartum phase in pregnant women. *Y*-axis represents the media values. Mann–Whitney statistical test was used for calculation of the reported *p*-value. Also, $p < 0.05$ was considered to be statistically significant. Statistical significance is marked as $^*p < 0.05$ and $^{**}p < 0.001$.

gender: when comparing with healthy men, MS men showed a significantly increased activation of both cytCD56^{dim} NK and CD3$^+$CD56$^+$CD8$^+$ cells. The most activated subset was cytCD56^{dim}, with a fourfold increase in CD69 expression [11.34 (5.45–33.94) vs. 2.76 (1.89–7.90), $p = 0.013$]. Also, MS men had significantly higher activated CD3$^+$CD56$^+$CD8$^+$ cells than healthy men [8.39 (5.89–27.02) vs. 3.93 (2.04–6.53), $p = 0.017$] (**Figure 4**), suggesting its relevance in MS pathophysiology.

For women, MS women disclosed significantly higher proportions of the regCD56^{bright} subset compared to healthy women [0.54 (0.42–0.78) vs. 0.33 (0.23–0.58), $p = 0.003$] (**Figure 5**). There were no significant differences in cytNK and CD3$^+$CD56$^+$CD8$^+$ cells' proportions between both MS and healthy groups.

We also evaluated the balance between cytotoxic vs. regulatory NK cells through the cytCD56^{dim}/regCD56^{bright} ratio and the cytCD56^{dim}/CD3$^+$CD56$^+$CD8$^+$ cells ratio. We found significantly increased ratios of CD69 expression in MS women with respect to healthy women (cytCD56^{dim}/CD3$^+$CD56$^+$CD8$^+$, $p < 0.001$; cytCD56^{dim}/regCD56^{bright}, $p = 0.06$) (**Figure 6**).

DISCUSSION

The most remarkable finding was the significant increase in activation of blood CD3$^+$CD56$^+$CD8$^+$ cells in MS patients during pregnancy with respect to NPMS and even above that of the healthy pregnant women. In addition, healthy full-term pregnancies showed also significantly higher activation of CD3$^+$CD56$^+$CD8$^+$ cells with respect to healthy non-pregnant women. The immunology of pregnancy is characterized by an anti-inflammatory shift paradigm in peripheral blood based on CD4$^+$ Treg and Th differentiation toward Th2 and regulatory CD4$^+$ T cells (Treg) (30–32). Changes in hormone levels during pregnancy, such as increased estrogens and progesterone, may

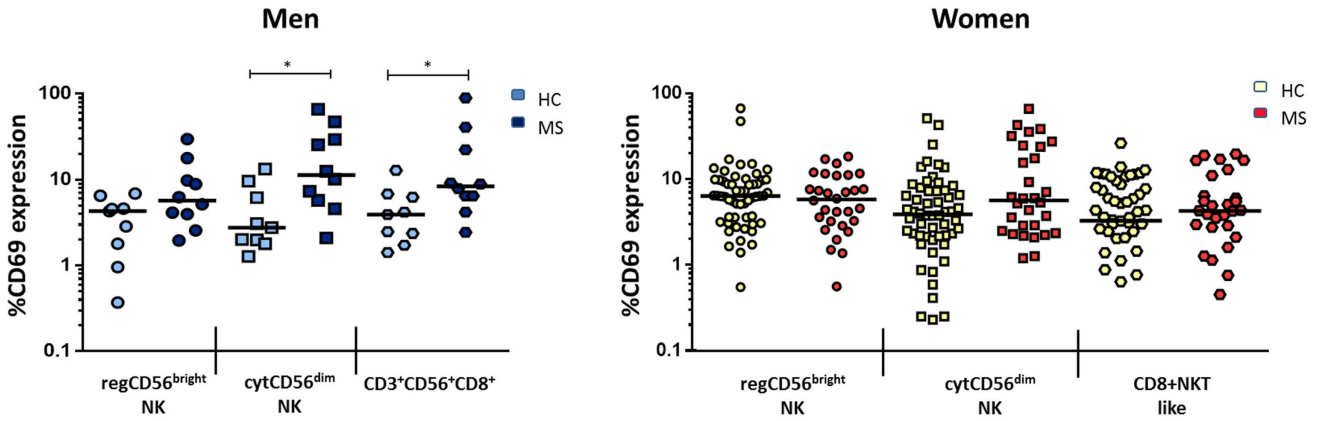

FIGURE 4 | Expression of CD69 (percentage) of natural killer (NK) subsets regCD56^{bright} (CD3$^-$CD56$^+$CD16$^-$), cytCD56^{dim} (CD3$^-$CD56$^+$CD16$^+$), and CD3$^+$CD56$^+$CD8$^+$ cells in men (left) [HC: $n = 9$, multiple sclerosis (MS): $n = 10$] and women (right) (HC: $n = 32$, MS: $n = 30$) MS compared to healthy individuals. Mann–Whitney statistical test was used for calculation of the reported p-value. The bar represents the median value, and individual dots indicate single donor values. Also, $^*p < 0.05$ was considered to be statistically significant.

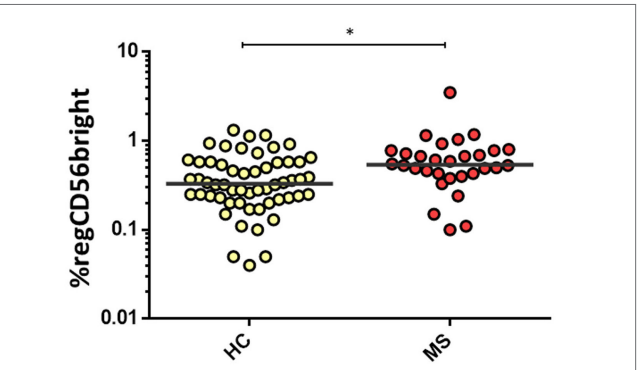

FIGURE 5 | Percentage of regCD56^{bright} in women from HC ($n = 32$) and multiple sclerosis (MS) ($n = 30$) groups. The bar represents the median value, and individual dots indicate single donor values. Mann–Whitney statistical test was used for calculation of the reported p-value. Also, $^*p = 0.003$ was considered to be statistically significant.

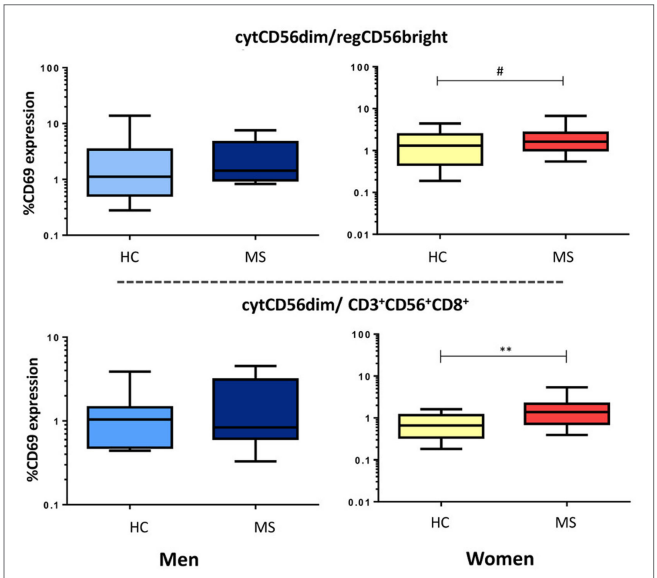

FIGURE 6 | Ratio of cytCD56^{dim} and regulatory cell subsets (regCD56^{bright} or CD3$^+$CD56$^+$CD8$^+$) activity in multiple sclerosis (MS) men (HC: $n = 9$, MS: $n = 10$) and women (HC: $n = 32$, MS: $n = 30$). Data are plotted with box and whiskers (10–90% range), and the bar represents the median value. Also, $p < 0.05$ was considered to be statistically significant. Statistical significance is marked as $^{**}p < 0.001$ and $^{\#}p = 0.06$ (trend).

be responsible for these changes. Thus pregnancy, through the promotion of an immunoregulatory status, has been related to have the most beneficial effects on the severity of several Th1/Th17-driven autoimmune diseases (33).

No reliable studies have shown the role of NKT in the setting of pregnancy and in MS, although our data suggest a potential role of this activated regulatory subset in the pregnancy-related remission of disease activity. Here, we observe that the highest activation status of CD3$^+$CD56$^+$CD8$^+$ cells occurred at the third trimester of gestation, when sex hormones reach their peak, and thereby would coincide with the reported strongest decrease in relapse rate in MS patients (34). Further, persistent although lower activation of CD3$^+$CD56$^+$CD8$^+$ cells was observed at PP, when the probability of MS relapse increases. The pattern of activation of CD3$^+$CD56$^+$CD8$^+$ cells was quite similar to that reported by our group and others for Treg, which have a peak during the second and third trimesters and declining at PP (35), which could reflect their possible implication in MS activity during pregnancy and PP. Regulatory function of CD8$^+$NKT cells has been previously reported in several disease mouse models characterized by Th1 immune responses through the production of a Th2-type cytokine profile (24, 36). The changes observed here, especially the high activation status of the regulatory subsets, lead us to speculate that CD8$^+$NKT cells could play an important role in modulating T cell responses and in ameliorating MS during pregnancy.

Epidemiological and clinical data clearly underline the sexual dimorphism in MS incidence and disease course. This fact directs to the influence of hormones on immune cells, and estrogens are known to exert opposing and dose-dependent effects on the immune response. There is controversy about specific effects of sex hormones on NK activity (37). In one hand, low estrogen levels facilitate a cell-mediated proinflammatory immune response, whereas their relatively high levels (pregnancy) promote anti-inflammatory Th2 and Treg responses (38). Our findings showed no differences between the follicular and ovulatory phases in the activity or proportions of the three NK and $CD3^+CD56^+CD8^+$ populations studied in healthy subjects. Concerning gender-based differences, our results showed no differences in peripheral NK or $CD3^+CD56^+CD8^+$ cell numbers or activity between women and men in healthy and MS groups. Moreover, we did not observe correlation among sex hormones estradiol and progesterone with NK and $CD3^+CD56^+CD8^+$ cells' subsets. Reported studies on the fluctuation of NK cells subsets during the menstrual cycle are heterogeneous, showing no changes during the cycle, increase, or even reduction in the luteal phase (37, 39). These results may in part be due to varying definitions of the NK populations and methodologies to define these cells and to measure activity.

However, differences between MS patients and healthy controls differentiated by gender were remarkable. Our findings showed increased activation of the cytotoxic $CD56^{dim}$NK subset in men affected of MS with respect to healthy men. These major increase of the cytotoxic NK subset studied would be compatible with previous results reporting increased circulating cyt$CD56^{dim}$ NK levels expressing perforin in primary progressive MS patients, a clinical form with men' predominance (40), which might suggest a role of this NK cell subset in the pathophysiology and progression of the disease. The increased activation of the regulatory $CD3^+CD56^+CD8^+$ subset reported in our series of MS men could be reflecting a compensatory activation to control the inflammatory activity. On the other hand, MS women show an increased proportion of reg$CD56^{bright}$ NK cells compared to healthy women. Thus, the increased activation of the cytotoxic $CD56^{dim}$ NK subset in men, and the increased proportion of reg$CD56^{bright}$ NK cells in women, would be in line with the sex differences reported in the clinical course of MS. Although prevalence in women is higher, there is evidence that women generally have an earlier onset of disease, slightly lower prevalence of PP-MS, and show in general less progression of disability than men (41).

Particular NK cell subtypes may have different roles in controlling CNS inflammation in MS patients, and the balance between immunity and tolerance would be orchestrating the adaptive autoreactive response. MS men showed an increased ratio of cytotoxic NK vs. regulatory NK/NKT cells' activation; whereas MS women showed a significantly increased proportion reg$CD56^{bright}$ NK cells than healthy women. However, the immune balance between cytotoxic and regulatory NK cells was highly increased in MS patients, more marked in women. In terms of NK cells' functions, we could speculate that more than a defective regulatory response, an imbalance due to enhanced cytotoxic activity could contribute to the MS pathological processes.

Understanding the distribution and function of NK and NKT cells' subsets related to gender disparity and the effects during pregnancy status in MS may help to have a global vision and to fully understand the implication of innate immunity on MS. Differences observed and beneficial effects of pregnancy in MS highlight the relevance of NKT cells. Further studies on $CD8^+$ NKT cells function and their role in pregnancy beneficial effects on MS are warranted to move forward more effective MS treatments. NKT-like cells would be potential therapeutical targets in MS as well as therapies that enhance their numbers or activation.

ETHICS STATEMENT

All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Ethical and Scientific Committees of the Hospital.

AUTHOR CONTRIBUTIONS

CA recruited and followed patients, wrote the draft the manuscript, and performed critical revision; LF-P analyzed data, wrote the draft the manuscript and figures; BA acquired data; MT-A and RR-M acquired data, analyzed and revised the manuscript; SS-R designed concept of the research and experiments, analyzed data and critical revision, and wrote the manuscript.

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CD28⁻ and CD28^{low}CD8⁺ Regulatory T Cells: Of Mice and Men

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Since the rebirth of regulatory (formerly known as suppressor) T cells in the early 1990s, research in the field of immune-regulation by various T cell populations has quickly gained momentum. While T cells expressing the transcription factor Foxp3 are currently in the spotlight, several other T cell populations endowed with potent immunomodulatory capacities have been identified in both the CD8⁺ and CD4⁺ compartment. The fundamental difference between CD4⁺ and CD8⁺ T cells in terms of antigen recognition suggests non-redundant, and perhaps complementary, functions of regulatory CD4⁺ and CD8⁺ T cells in immunoregulation. This emphasizes the importance and necessity of continuous research on both subpopulations of regulatory T cells (Tregs) so as to decipher their complex physiological relevance and possible synergy. Two distinct CD8-expressing Treg populations can be distinguished based on expression of the co-stimulatory receptor CD28. Here, we review the literature on these (at least in part) thymus-derived CD28^{low} and peripherally induced CD28⁻CD8⁺ Tregs.

Keywords: tolerance, regulatory T cells, CD8⁺ T-lymphocytes, human, mouse, thymus, immunoregulation

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INTRODUCTION

The prerequisite to the prevention of immunopathologies such as autoimmunity and chronic inflammation is the maintenance of an immune homeostasis that relies mainly on intricate mechanisms of tolerance to self and innocuous non-self antigens. Through their multifaceted actions, regulatory T cells (Tregs) play an unparalleled role in modulating both innate and adaptive responses. As such, Tregs prevent autoimmune disorders, control immune reactions at environmental surfaces, modulate anti-infectious responses, and contribute to fetomaternal tolerance [reviewed in Ref. (1–3)].

Historically speaking, the first suppressor population to be described were T cells expressing the CD8 co-receptor. Indeed, the T cell population identified by Cantor et al., which act in an antigen-specific manner to suppress immune reactions, expressed the surface marker Lyt2, now known as CD8α (4, 5). Since then, the field has had its fair share of whirls on the wheel of scientific (mis)fortune. The concatenation of events from the downfall of suppressor T cells to its rebirth (or

Abbreviations: AIRE, autoimmune regulator; APC, antigen-presenting cells; APECED, autoimmune polyendocrinopathy candidiasis ectodermal dystrophy; APS, autoimmune polyglandular syndrome; EAE, experimental autoimmune encephalomyelitis; Foxp3, forkhead/winged helix transcription factor; GFP, green fluorescent protein; IFN-γ, interferon-γ; IPEX, immune dysregulation polyendocrinopathy enteropathy X linked; ILT, immunoglobulin-like transcript; RAG, recombinase activating gene; SCID, severe combined immunodeficiency; TGF-β, transforming growth factor β.

rebranding) as Tregs have extensively been reviewed elsewhere (6–8) and will not be discussed here.

The advent of molecular immunology in the postsuppressor era unequivocally established the T cell population expressing the forkhead/winged helix transcription factor Foxp3 as a key player in the fine regulation of immune responses [reviewed in Ref. (9)]. Indeed, in mice, invalidating mutations in the *Foxp3* gene or specific ablation of Foxp3^{+} T cells lead to the development of a fatal lymphoproliferative disorder (10–13) and humans with mutations in the FOXP3 gene suffer from the lethal immune-dysregulation polyendocrinopathy enteropathy X linked syndrome (14, 15). In parallel, several other regulatory CD4^{+} and CD8^{+} subsets have been identified and characterized in both mice and humans (16–20). Distinct Treg (sub)populations differ in their origin, development, and mechanisms of action which *in fine* define their physiological role. As such, determining the specific function of a given Treg population mandates extensive research to identify the different molecular and cellular factors that govern its existence. We and others have contributed to unveil some key features of the CD8-expressing Treg population that is characterized by the expression of low levels of the co-stimulatory molecule CD28; CD8^{+}CD28^{low} Treg.

CD8^{+}CD28^{low} TREG IN MICE

The immunosuppressive capacity of CD8^{+}CD28^{low} was first described in a murine model of multiple sclerosis. Najafian et al. showed that CD8 knockout (CD8 KO) mice were more susceptible to the induction of experimental autoimmune encephalomyelitis (EAE) than wild-type (WT) mice suggesting a protective effect of CD8^{+} cells. Adoptive transfer of CD8^{+}CD28^{low} T cells from WT animals into CD8 KO recipients significantly reduced the severity of the disease. No such decrease was observed with the adoptive transfer of CD8^{+}CD28^{high} T cells. Furthermore, CD8^{+}CD28^{low} T cells but not their CD28^{high} counterpart could suppress *in vitro* the production of interferon-γ by CD4^{+} T cells specific for the myelin oligodendrocyte glycoprotein used to induce EAE. The suppressive function of the CD8^{+}CD28^{low} Treg required an interaction with antigen-presenting cells (APC), which led to the downregulation of CD80, CD86, and CD40 expression on the APC (21). In a similar model, Yang et al. have shown that pretreatment of mice with a group of 15-amino acid-long trichosanthin-derived peptides reduced the clinical score of EAE as compared to untreated animals. Attenuation of the disease was attributed to the expansion and activation of IL10-producing-CD8^{+}CD28^{low} Treg (22).

Previous work by our team has shown that CD8^{+}CD28^{low} Treg can prevent intestinal inflammation in a well-established experimental colitis model where pathology is induced by the adoptive transfer of naïve CD4^{+}CD45RB^{high} T cells into lymphopenic animals [recombinase activating gene 2 (RAG2) deficient or severe combined immunodeficiency mice (23, 24)]. Cotransfer of freshly isolated splenic CD8^{+}CD28^{low} T cells from WT animals with the colitogenic cells prevented onset of colitis. Similar results were obtained with CD8^{+}CD28^{low} T cells isolated from the lamina propria of the intestine (25). These CD8αβ^{+}CD28^{low} Treg expressed a large repertoire of the TCRαβ heterodimer (26). Protection from colitis was dependent on IL-10 production by the Treg and on the responsiveness of the colitogenic T-cells to transforming growth factor β (TGF-β), underlining the non-redundant functions of these two immunomodulatory cytokines in the control of intestinal inflammation by CD8^{+}CD28^{low} Treg (25). Importantly, in contrast to CD4^{+}CD25^{high} Treg, CD8^{+}CD28^{low} Treg from unmanipulated mice do not express the transcription factor Foxp3. More recently, in mice immunized with ovalbumin and subsequently intranasally challenged with ovalbumin encased in oligomannose-coated liposomes, an expansion of CD8^{+}CD28^{low} (and CD4^{+}Foxp3^{+}) Treg was observed. Upon adoptive transfer, the CD8^{+}CD28^{low} Treg reduced the severity of allergic diarrhea (27).

AUTOIMMUNE REGULATOR (AIRE) AND THE DEVELOPMENT OF CD8^{+}CD28^{low} TREG

The transcription factor AIRE is primarily expressed by medullary epithelial cells of the thymus (mTEC) where it controls cellular maturation and the ectopic expression of thousands of tissue-specific antigens (28, 29). Presentation of these peripheral antigens by mTEC leads to the negative selection of auto-specific conventional T cells (30–32). Furthermore, AIRE modulates the production of chemokines by mTEC, involved in the migration of thymocytes and dendritic cells from the cortex to the medulla in the thymus (33, 34). As such, AIRE is a key regulator of central tolerance. Indeed, loss-of-function mutations in the AIRE gene lead to the autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED) syndrome also known as APS for autoimmune polyglandular syndrome (35, 36). While chronic mucocutaneous candidiasis, hypoparathyroidism, and hypoadrenalism are considered to be the classic triad hallmarks of this autoimmune syndrome (37), about 25% of APECED patients are also affected by gastrointestinal diseases ranging from chronic diarrhea and obstipation (38). In children suffering from APECED, these intestinal ailments can lead to malabsorption, various deficiencies, growth impairment, and even death (39, 40). Importantly, some do even consider gastrointestinal symptoms to be the first manifestation of APECED (38). Mice deficient for AIRE also exhibit (though to a lesser extent) autoimmune symptoms such as presence of autoantibodies and cellular infiltration in various organs (41). Since CD8^{+}CD28^{low} Treg can efficiently prevent intestinal inflammation, a prominent symptom in APECED, the potential role of AIRE in the development of this Treg population was evaluated.

Our comparative study of CD8^{+}CD28^{low} Treg from WT and AIRE-deficient (AIRE KO) mice revealed that while both Treg populations were present in similar proportions and exhibited comparable immunosuppressive activity *in vitro*, Treg from AIRE KO animals failed to prevent intestinal inflammation in the colitis model (26). Gene expression patterns, cell-surface marker expression, IL-10 production, and *in vitro* suppressive capacity of WT and AIRE KO CD8^{+}CD28^{low} Treg were indistinguishable. However, a small difference was found between the T-cell receptor (TCR) repertoires expressed by WT vs. KO

Treg. Based on these observations, we concluded that AIRE is involved in shaping the TCR-repertoire of CD8^{+}CD28^{low} Treg. To our knowledge, this was the first definite demonstration that a deficiency in AIRE leads to the functional defect of a Treg population. This pioneer study is in line with more recent studies that have provided molecular evidence, through TCR repertoire analysis, that AIRE is essential for the thymic development of CD4^{+}Foxp3^{+} Treg with unique individual TCRs (42–44). Taken together, these studies have established that AIRE not only drives the negative selection of conventional T cells but is also involved in the differentiation of CD8^{+} and CD4^{+} Treg populations.

ORIGIN OF CD8^{+}CD28^{low} TREG

Based on our current understanding of the development of CD4^{+}Foxp3^{+} Treg, it is commonly accepted that Treg in general can have two distinct origins: intrathymic development of "tTreg" from hematopoietic precursors and extrathymic (or peripheral) differentiation of "pTreg" from conventional T cells given appropriate environmental cues [reviewed in Ref. (45, 46)]. Since data from the literature have attributed distinct singular functions to tTreg and pTreg (47–49), the identification of the origin of CD8^{+}CD28^{low} Treg was an important milestone in the quest to better characterize this population. Our observation that AIRE, which is primarily expressed in the thymus, is involved in the development of the CD8^{+}CD28^{low} Treg repertoire suggested a thymic origin for CD8^{+}CD28^{low} Treg. However, expression of AIRE has also been reported in both hematopoietic and stromal lineages outside of the thymus (50–52). Importantly, these extrathymic AIRE-expressing cells have tolerogenic properties (53) and thus in theory may induce differentiation of conventional T cells into Tregs. We recently demonstrated that mature CD4^{-}CD8^{+}TCR^{high} thymocytes expressing low levels of CD28, isolated from WT mice, can efficiently suppress the *in vitro* proliferation of CD4^{+} T cells (54). However, since T cells including Tregs can recirculate from the periphery back to the thymus (55, 56), their presence in this primary lymphoid organ was not sufficient to confirm their origin. Definite proof of the thymic origin of CD8^{+}CD28^{low} Treg came from the analysis of transgenic mice expressing the green fluorescent protein (GFP) under the control of the RAG2 promoter [RAG–GFP mice, Ref. (57)]. In the thymus, thymocytes express RAG2 at the early stages of their development and then terminate its expression after positive selection (58). As such, in RAG–GFP animals, the GFP protein whose expression parallels that of RAG2 and has a half-life of 56 h serves as a molecular marker for lymphocyte aging in the thymus allowing for the discrimination between "freshly" developed mature T cells that express GFP and recirculating T cells that do not (59). Analysis of RAG–GFP mice revealed that while approximately 20% of mature thymic CD8^{+}CD28^{low} T cells are deprived of GFP expression (i.e., recirculating or long-term thymus resident cells), the major proportion of this T cell population are newly developed cells. Importantly, the GFP^{+} compartment of the mature thymic CD8^{+}CD28^{low} T cells demonstrated immunosuppressive activity *in vitro* hence firmly establishing the thymic origin of CD8^{+}CD28^{low} Treg in mice (54). However, the interesting possibility that the pool of circulating CD8^{+}CD28^{low} Treg may be composed of both tTreg and pTreg must also be considered. Indeed, in an experimental model of myasthenia gravis (MG), exposure to specific antigens (the dual-altered peptide) led to the emergence of CD8^{+}CD28^{low} Treg (60). While it can be argued that the emergence of Treg could be due to the expansion of preexisting tTreg, the alternate hypothesis of an induction of *bona fide* pTreg cannot be excluded (**Figure 1**).

CD8^{+}CD28^{low} TREG IN HUMANS

A population of CD8^{+}CD28^{low} T cell exhibiting similar immunosuppressive characteristics as its murine homolog has recently been identified in humans. Analysis of peripheral blood mononuclear cells (PBMCs) has revealed a substantial percentage (between 10 and 13%) of CD28^{low}-expressing cells among naive CD8^{+} T cells. Importantly, following *in vitro* activation, these cells produce the same cytokines (i.e., IL-10 and TGF-β), which confer CD8^{+}CD28^{low} Treg their immunomodulatory ability in experimental mouse models. Similar results were obtained when human thymii isolated from children aged from 0 to 10 years were analyzed (54). Taken together, these results from human studies strongly suggest that, similar to mouse, CD8^{+}CD28^{low} T cell endowed with immunosuppressive capacity are present in human PBMCs and that they develop in the human thymus.

CD8^{+}CD28^{-} TREG IN HUMANS AND MICE

Based on CD28 expression, another Treg population has previously been described in humans. Cyclic stimulations of PBMCs with allogenic APC induced CD8^{+} T cells deprived of CD28 expression, which inhibited cellular proliferation in these *in vitro* cultures (61). Since then, several groups have tried to develop, with more or less success, their own strategies to induce CD8^{+}CD28^{-} Treg *in vitro* by stimulating PBMCs with cocktails of cytokines in the presence or absence of antigens (62, 63), with phorbol12-myristate 13-acetate/ionomycin or phytohemagglutinin (64) or with a recombinant immunoglobulin-like transcript 3 (ILT3)-Fc fusion protein (65, 66). CD8^{+}CD28^{-} Tregs express GITR, CD25, CD103, CD62L, and 4-IBB and are MHC class I restricted (67). They exert their immunomodulatory activity by inducing the expression of ILT3 and ILT4 on dendritic cells, thus rendering them tolerogenic (66). Intriguingly, human mesenchymal stromal cells have recently been shown to enhance the immunomodulatory function of CD8^{+}CD28^{-} Treg by reducing their rate of apoptosis (68).

CD8^{+}CD28^{-} T cells with a regulatory phenotype have been observed in patients having undergone successful organ transplantation (69–71), alloanergized HLA-mismatched bone marrow graft (72), and allogenic platelet transfusion (73) or suffering from autoimmune diseases (74–76), pregnancy complications (77), and cancers (78–80). Importantly, CD8^{+}CD28^{-} T cells isolated from healthy donors are not immunosuppressive (69). Hence, it would seem that CD8^{+}CD28^{-} Treg are induced in the periphery following disturbances of the immune homeostasis.

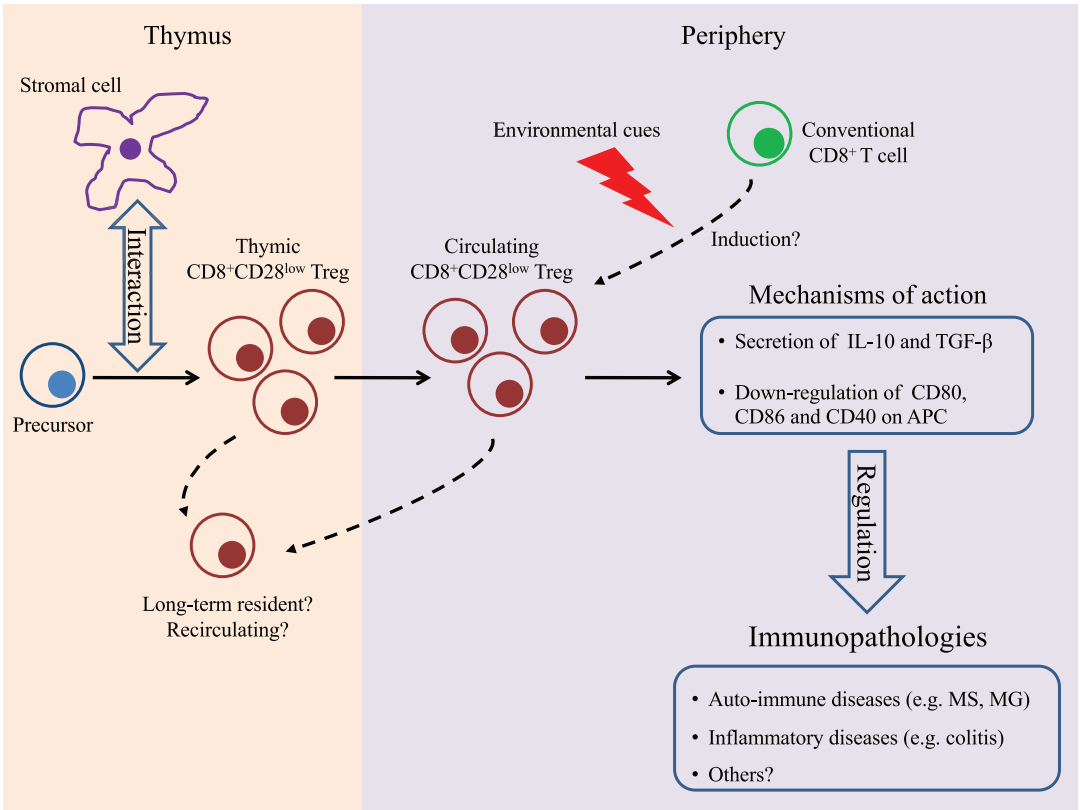

FIGURE 1 | Summary of findings on CD8⁺CD28^{low} regulatory T cells (Tregs). In the thymus, T cell precursors will interact with stromal cells presenting antigens that are expressed under control of the transcription factor autoimmune regulator and differentiate into tTreg. In the periphery, these cells will enforce their regulatory function by secreting immunomodulatory cytokines and/or by inhibiting antigen-presenting cells. It cannot be excluded that CD8⁺CD28^{low} Treg can also differentiate, under specific tolerogenic conditions, in the periphery. So far, the immunosuppressive capacity of CD8⁺CD28^{low} Tregs has been documented in experimental models of multiple sclerosis, myasthenia gravis, and colitis. Solid lines indicate established features, and dashed lines indicate potential characteristics.

A mouse homolog of human CD8⁺CD28⁻ pTreg may also exist. Ben-David et al. showed that in an experimental model of MG where pathology is triggered by immunization with a myasthenogenic peptide, injection of a dual-altered peptide induces the emergence of CD8⁺CD28⁻ Treg that efficiently suppress the autoimmune response. Flow cytometry analysis of these cells suggested that these Tregs may express low levels of Foxp3 (60).

CD28⁻ VS. CD28^{low}CD8⁺ TREG IN HUMANS AND MICE

Najafian et al. initially showed that total CD8⁺ T cells isolated from CD28-deficient mice (i.e., CD8⁺CD28⁻ cells) exhibited immunosuppressive activity *in vitro* and decreased the severity of EAE in adoptive transfer experiments. However, the CD8⁺ T cells isolated from WT mice that inhibited severity of EAE clearly expressed low levels of CD28 (21). In our initial report on the prevention of experimental colitis in the mouse, the CD8⁺ Treg, which we inaccurately termed CD28⁻, also clearly expressed low but detectable levels of CD28. In unmanipulated specific pathogen-free WT mice, we only observed subsets of CD8⁺ T cells expressing low or high levels of CD28 but none that are deprived of expression of this co-stimulatory molecule (25, 26, 54). In humans, their low but readily detectable level of expression of CD28, their presence in the thymus, and their naive phenotype clearly distinguish CD8⁺CD28^{low} Treg from CD8⁺CD28⁻ Treg that do not express CD28 at levels exceeding background, are not found in the thymus, and have an activated phenotype (54). We therefore conclude that the co-stimulatory molecule CD28 allows for the identification of two distinct CD8⁺ subsets: CD28^{low} tTreg and CD28⁻ p Treg.

CONCLUDING REMARKS

While the various studies discussed here have helped to decipher key features of CD8⁺CD28^{low} T cells and in parallel establish them as a potent Treg population in both mice and humans, several burning questions concerning these Treg remain unanswered, the most important one being perhaps their biological function(s) under homeostatic and pathologic conditions. We believe that the identification of other, more discriminative, markers of CD8⁺CD28^{low} Treg will greatly help in achieving this goal. Currently, this Treg population can only be characterized by their low levels of expression of CD28 allowing for only a minimal

estimation of their proportions by flow cytometry analysis (25, 26, 54). Furthermore, the absence of a better marker is hindering a panoply of key experiments such as specific localization in tissues and lymphoid organs, antibody-specific depletion, germline, and/or conditional knockout strategies.

Up till now, research on CD8^{+}CD28^{low} Treg had been confined to murine studies (21, 25, 26). Even though the potent immunoregulatory capacity of CD8^{+}CD28^{low} has been documented in these experimental models of inflammation, its relevance in human diseases remains unknown. In parallel, defects in various CD4^{+} and CD8^{+} Treg populations have been reported in human autoimmune diseases and immune-mediated inflammatory pathologies (81–87). The identification of CD8^{+}CD28^{low} Treg in humans is hence paving the way to further studies so as to gain insight into the physiological function of this Treg population and its potential involvement in human pathologies.

AUTHOR CONTRIBUTIONS

YV and JPMvM designed the outline and wrote the manuscript.

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The Hypothesis of the Human iNKT/Innate CD8(+) T-Cell Axis Applied to Cancer: Evidence for a Deficiency in Chronic Myeloid Leukemia

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We recently identified a new human subset of NK-like [KIR/NKG2A(+)] CD8(+) T cells with a marked/memory phenotype, high Eomesodermin expression, potent antigen-independent cytotoxic activity, and the capacity to generate IFN-γ rapidly after exposure to pro-inflammatory cytokines. These features support the hypothesis that this new member of the innate T cell family in humans, hereafter referred to as innate CD8(+) T cells, has a role in cancer immune surveillance analogous to invariant natural killer T (iNKT) cells. Here, we report the first quantitative and functional analysis of innate CD8(+) T cells in a physiopathological context in humans, namely chronic myeloid leukemia (CML), a well-characterized myeloproliferative disorder. We have chosen CML based on our previous report that IL-4 production by iNKT cells was deficient in CML patients at diagnosis and considering the recent evidence in mice that IL-4 promotes the generation/differentiation of innate CD8(+) T cells. We found that the pool of innate CD8(+) T cells was severely reduced in the blood of CML patients at diagnosis. Moreover, like iNKT and NK cells, innate CD8(+) T cells were functionally impaired, as attested by their loss of antigen-independent cytotoxic activity and IFN-γ production in response to innate-like stimulation with IL-12 + IL-18. Remarkably, as previously reported for IL-4 production by iNKT cells, both quantitative and functional deficiencies of innate CD8(+) T cells were at least partially corrected in patients having achieved complete cytogenetic remission following tyrosine kinase inhibitor therapy. Finally, direct correlation between the functional potential of innate CD8(+) T and iNKT cells was found when considering all healthy donors and CML patients in diagnosis and remission, in accordance with the iNKT cell-dependent generation of innate CD8(+) T cells reported in mice. All in all, our data demonstrate that CML is associated

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with deficiencies of innate CD8(+) T cells that are restored upon remission, thereby suggesting their possible contribution to disease control. More generally, our study strongly supports the existence of an innate iNKT/innate CD8(+) T-cell axis in humans and reveals its potential contribution to the restoration of tumor immune surveillance.

Keywords: innate CD8(+) T cells, NK-like CD8(+) T cells, iNKT cells, chronic myeloid leukemia, tyrosine kinase inhibitor

INTRODUCTION

A hallmark of the antigen-specific T lymphocytes of the adaptive immune system is their capacity to "remember" foreign pathogens long after they are first encountered. Indeed, as far as the CD8 T cell pool is concerned, the main features that provide antigen-specific memory CD8(+) T cells with a protective advantage over naive CD8+ T cells are that memory CD8+ T cells persist for extended periods of time, are present in larger numbers, and respond more rapidly to foreign antigen than naive CD8(+) T cells do. However, studies in mice have provided clear evidence that some naive CD8(+) T cells can acquire the characteristics and functions of memory CD8(+) T cells in the absence of foreign antigen encounters [reviewed in Ref. (1, 2)]. Such cells, hence called innate/memory CD8(+) T cells, may develop in response to alterations in the environment or the presence of high levels of the cytokine IL-4, and have also been identified in unmanipulated animals. A hallmark of innate/memory CD8(+) T cells is their marked NK-like/memory phenotype associated with pronounced expression of the transcription factor eomesodermin (Eomes) (3, 4). Even though the actual physiological significance of innate/memory CD8(+) T cells remains to be established, these cells have enhanced response potential, including the ability to efficiently combat pathogens (5, 6). Moreover, because these cells can rapidly produce large amounts of IFN-γ in response to innate-like stimulation by IL-12 + IL-18 (7, 8), they might contribute to the inflammation milieu during an early stage of immune response.

The existence of innate/memory CD8(+) T cells in mice raises the question whether an equivalent to these innate T cells exists in humans. Earlier studies identified a subset of CD8(+) T cells in human peripheral blood that express NK-cell receptors such as killer cell Ig-like receptors (KIRs) (9–11). We further demonstrated that the majority of KIR/NKG2A(+) CD8(+) T cells have a memory phenotype and share functional and phenotypic features (12), see commentary (13): with innate/memory T CD8(+) cells in mice (3, 4, 7, 8). Indeed, they express high levels of Eomes and display innate functions, such as cytolytic capacity and rapid TCR-independent IFN-γ production in response to the innate cytokines IL-12 and IL-18. Regardless of their origin, KIR/NKG2A(+) Eomes(+) CD8(+) T cells harboring memory phenotype and innate-like functions have also been identified in human cord blood, suggesting that their development did not depend on cognate antigens. Additionally, the presence of these cells correlated well with expression of promyelocytic leukemia zinc finger (PLZF) among NKT cells in cord blood, a finding that is consistent with the hypothesis of a contribution of IL-4-producing, PLZF-expressing cells to their generation, as reported for their equivalents in mice (14, 15). These features validate the existence of a new member of the innate T cell family in humans, which we have termed innate CD8(+) T cells. However, to date, even though evidence accounts for their potential physiological relevance, whether innate CD8(+) T cells can mediate potent immunity in humans remains to be investigated.

Chronic myeloid leukemia (CML) is a well-characterized myeloproliferative disorder, which results from dysregulated tyrosine kinase (TK) activity of the fusion oncoprotein BCR-ABL (16). Imatinib mesylate (IM), a competitive inhibitor of the BCR-ABL TK activity, is currently used as a first-line therapy for newly diagnosed patients in the chronic phase (CML-CP) (17). Overall, TK inhibitor (TKI) therapies have led to deep molecular responses in CML, dramatically improved life expectancy, and more recently, successful treatment-free survival has become a new goal (18). However, a significant proportion of patients will not fulfill the conditions allowing for treatment discontinuation while more than 40% of those eligible for treatment discontinuation relapse during the first 6 months after therapy cessation. Additional therapeutic strategies aiming at a potentiation and/or restoration of immune antitumor functions, therefore, remain a main avenue of research, which may be helpful in long-term control of CML.

A sizable number of clinical and experimental data underscore the dysregulation of innate immune components in CML. These regulatory elements comprise dendritic cells (19), NK cells (20, 21), and invariant natural killer T (iNKT) cells (22), an innate T cell subset co-expressing activated/memory and NK markers, which is well-recognized for its antitumor activity (23–26). In accordance with this notion, we recently reported CML immune subversion of iNKT-cell activities in CML patients at diagnosis (22, 27), including reduced or suppressed expression of perforin, CD95L, and (PLZF), a transcription factor required for maintenance of iNKT cell functions (28, 29). Remarkably, these functional deficiencies were shown to have been partially repaired in patients having achieved complete cytogenetic remission (CCyR) following TKI therapy (22).

In mice, IL-4-producing/PLZF-expressing cells, including iNKT cell, have been demonstrated to regulate the generation of innate/memory CD8(+) T cells (14, 15). Considering the functional deficiencies of iNKT cells in CML-CP patients at diagnosis and the fact that IL-4 production by iNKT cells was partially restored in patients having achieved CCyR after TKI therapy (22), we surmised that innate CD8(+) T cells might also be altered during CML and be restored in CML patients with CCyR. We further attempted to correlate the presence of innate CD8(+) T cells with that of iNKT cells to estimate the possible implication in humans of the peripheral iNKT cell pool in the development of its CD8(+) T-cell counterpart.

MATERIALS AND METHODS

Peripheral Blood Mononuclear Cells (PBMCs)

Venous blood from CML-CP patients at diagnosis or having achieved a major molecular response and currently treated with IM (CML-IM) was collected on heparin (Oncology-Hematology Department, Poitiers, France). All patients gave informed consent in accordance with the Declaration of Helsinki for participation in the study, which was approved by the scientific committee of the INSERM CIC-1402 (Poitiers, France). Healthy donors (HDs) were volunteers from the Pôle Biologie Santé (Poitiers, France). PBMCs were isolated from blood samples by density gradient centrifugation (Histopaque®-1077, Sigma-Aldrich), resuspended in 90% fetal calf serum with 10% DMSO, and placed in a controlled rate freezer for cryopreservation at −80°C until use.

Cell Culture and Functional Assays

All cell cultures (1×10^6 cells/mL) were performed in RPMI 1640 medium supplemented with 10% heat-inactivated FCS and antibiotics. For IL-12 + IL-18 stimulation, PBMCs from HD or CML patients were seeded at 1×10^6 cells/mL into 24-well plates and incubated for 48 h with 20 ng/mL of each cytokine (R&D Systems). Golgistop (BD Biosciences) was added for the last 5 h of culture. CD107a degranulation assays were performed as previously described (12). Briefly, PBMCs were seeded into 96-well round (*U*) bottom culture plates, preincubated for 48 h with IL-15 (20 ng/mL, R&D Systems) prior to CD16 triggering, and Golgistop (BD Biosciences) was added in the last 4 h of culture. For IL-4 stimulation, PBMCs from HDs were seeded at 0.5×10^6 cells/mL into 24-well plates and incubated for 7 days with 20 ng/mL of recombinant human IL-4 (R&D Systems).

Flow Cytometry

Phenotypic analysis of PBMCs was performed by flow cytometry either *ex vivo* or after culture. Expression of different markers was assessed by staining with appropriate combinations of the following antibodies (mAbs): anti-CD3 BV421 (clone: UCHT1, BioLegend), anti-CD8 PE-Cy7 (clone: RPA-T8, Biolegend), anti-IFN-γ FITC (clone: B27, BioLegend), anti-perforin FITC (clone: δG9, BD Biosciences), anti-TCR Vα24-Jα18 APC (clone: 6B11, Biolegend), anti-CD107a FITC (clone H4A3, BD Biosciences), anti-Eomes eFluor® 660 (clone: WD1928, eBiosciences), and anti-PLZF PE (clone: Mags.21F7, eBioscience). Pan-KIR/NKG2A referred to staining with the mix of the three following antibodies from Miltenyi Biotech: anti-KIR2D PE (clone: NKVFS1), anti-KIR3DL1/KIR3DL2 (CD158e/k) PE (clone: 5.133), and anti-NKG2A (CD159a) PE (clone: REA110). Dead cells were excluded by using the Live/Dead® Fixable Near-IR Dead Cell Stain kit (Life Technologies). For nuclear Eomes or PLZF staining and intracytoplasmic IFN-γ or perforin staining, cells were permeabilized with an anti-human Foxp3 staining kit (eBioscience) and a Cytofix/Cytoperm kit (BD Biosciences), respectively. Cells were analyzed by eight-color flow cytometry (FACSVerse™ cytometer and FACSuite™ software, BD Biosciences) and were analyzed using FlowJo v10 (TreeStar, Inc.). Innate CD8(+) T cells are defined as CD3(+) CD8(+) Eomes(+) KIR/NKG2A(+) and iNKT cells as CD3(+) TCRVα24-Jα18(+)-expressing cells after gating on live PBMCs.

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 6.0 (GraphPad Software). The statistical significance of differences in mean values was analyzed by the Mann–Whitney or Wilcoxon non-parametric test. The correlation Spearman test was used to test the association between the ranked variables Eomes and PLZF. Results were considered to be statistically significant when $p < 0.05$.

RESULTS

Quantitative and Functional Deficiencies of Innate CD8(+) T Cells from CML-CP Patients

CD8(+) T cells co-expressing Eomes and KIR/NKG2A represent a new, functionally distinct "innate" subset in humans, with potential antitumor activities (12, 13). Based on our evidence for CML immune subversion of iNKT-cell activities (22, 27), we first investigated possible dysfunctions of this new innate CD8 T subset (for gating strategy, see **Figure 1A**) in CML patients at diagnosis (CML-CP). As depicted in **Figure 1B**, the frequency of KIR/NKG2A(+) Eomes(+) CD8(+) T cells was more than 2.5-fold lower in CML-CP patients (3.1% ± 0.7; $n = 6$) than in HDs (8.2% ± 0.9; $n = 15$).

Note that both the proportion of cells expressing Eomes among the KIR/NKG2A(+) CD8(+) T cell subset (Figure S1 in Supplementary Material) and the expression levels (**Figure 1C**) were significantly reduced in CML-CP patients [frequency: 26.3% ± 3.2 ($n = 6$) and mean fluorescence intensity (MFI): 2.17 ± 0.25 ($n = 6$), respectively] as compared to HD [frequency: 46.5% ± 4.6 ($n = 14$) and MFI: 3.95 ± 0.52 ($n = 15$), respectively]. Taken together, these data are consistent with impaired differentiation of the innate CD8 T cell subset in CML-CP patients.

We have previously reported (12) and have confirmed in our present HD cohort (**Figure 2A**) that the rapid production of IFN-γ in response to innate-like stimulation by IL-12 + IL-18 constitutes a unique hallmark of innate CD8(+) T cells. Indeed, this functional reactivity to IL-12 + IL-18 was found in the innate KIR/NKG2A(+) Eomes(+) fraction (24.8% ± 1.2; $n = 6$) and not in the conventional/memory KIR/NKG2A(−) Eomes(+) pools of CD8(+) T cells (1.4% ± 0.8; $n = 6$).

Remarkably, this function was not maintained in innate CD8(+) T cells from CML-CP patients, in which IL-12 + IL-18-induced intracellular IFN-γ expression was virtually undetectable [0.7% ± 0.1 ($n = 6$) vs. 0.4% ± 0.02 ($n = 5$) in their conventional/memory counterpart], as compared with HD (24.8% ± 1.2 ($n = 6$) vs. 1.4% ± 0.8 ($n = 6$) in their conventional/memory counterpart) (**Figure 2A**). In HD, innate CD8(+) T cells express more cytolytic activity than does their conventional/memory [KIR/NKG2A(−) Eomes(+)] CD8(+) counterpart, as attested by their more elevated perforin expression [71.4% ± 5.3 ($n = 10$)

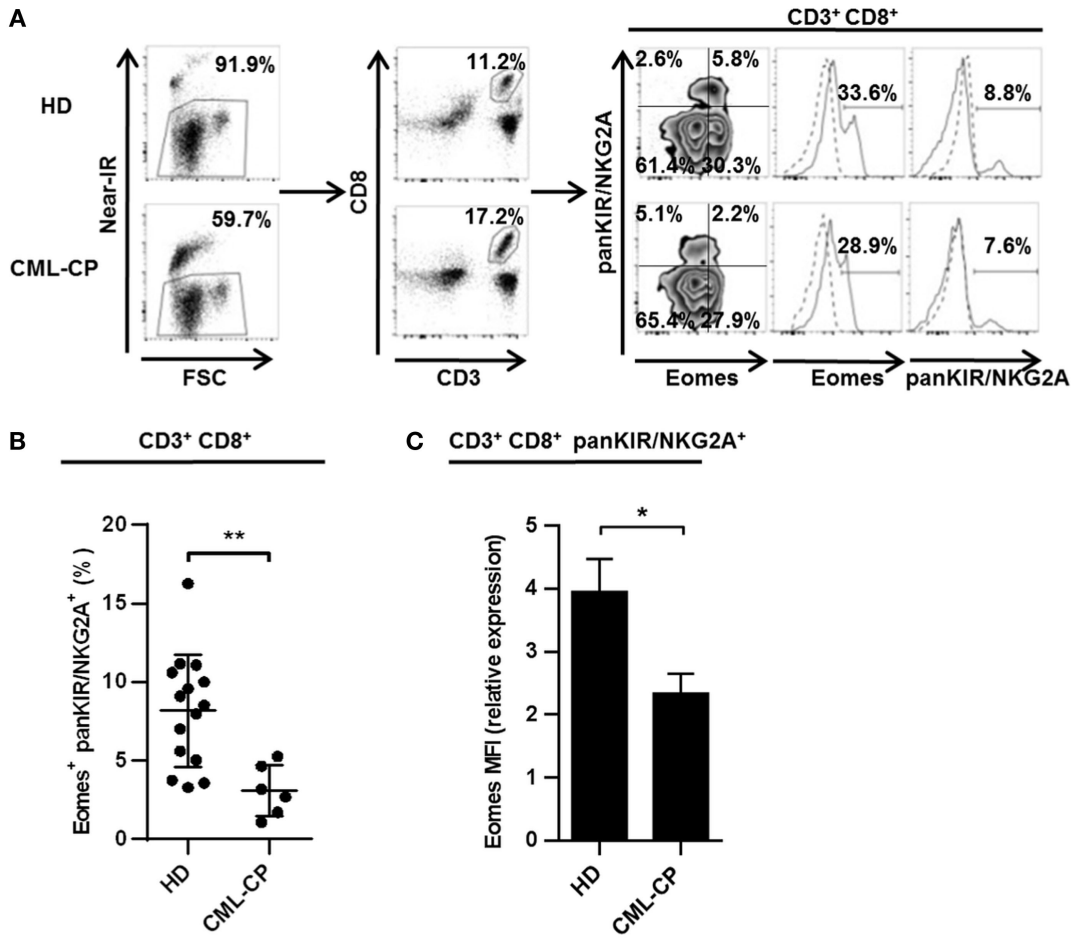

FIGURE 1 | Chronic myeloid leukemia (CML)-CP is associated with acquired quantitative defects of innate CD8(+) T cells. (A) Gating strategy. Eomes and killer cell Ig-like receptor (KIR)/NKG2A expression among peripheral blood mononuclear cells was analyzed by flow cytometry after gating on live cells (Near-IR) and then on CD8(+) CD3(+) populations. Quantile contour plots show one representative sample for each group of healthy donor (HD) or CML-CP patients. Solid and dotted lines on histograms represent Eomes or pan-KIR/NKG2A expression or isotype control, respectively. **(B)** Decreased innate CD8(+) T cell counts in CML-CP patients. Frequency (mean ± SEM) of KIR/NKG2A(+) Eomes(+) cells among total CD8(+) CD3(+) cells in HD and CML-CP patients. Each dot represents one HD or CML-CP patient. **(C)** Decreased Eomes expression in CD3(+) CD8(+) KIR/NKG2A(+) T cells in CML-CP patients. Mean fluorescence intensity (MFI) of Eomes expression (mean ± SEM) in KIR/NKG2A(+) CD8(+) T cells from HD and CML-CP patients were analyzed after gating on KIR/NKG2A(+) CD8(+) CD3(+) cells. MFI of Eomes expression was normalized on MFI of Eomes from total KIR/NKG2A(−) CD3(−) cells. Statistical significance was determined by the two-tailed Mann–Whitney non-parametric test. *$p < 0.05$; **$p < 0.01$.

and 42.4% ± 9.1 ($n = 6$), respectively] (**Figure 2B**), and natural antibody-dependent cytotoxicity through CD16 as a lysis receptor [26.4% ± 6.2 ($n = 6$) and 11.4% ± 3.5 ($n = 6$), respectively] (**Figure 2C**).

As for intracellular IFN-γ expression, these two functions were once again dramatically reduced in innate CD8(+) T cells from CML-CP patients [44.0 ± 6.7% ($n = 10$) and 6.8% ± 4.1 ($n = 5$), respectively] all the way down to the levels found in the conventional/memory population [26% ± 13 ($n = 6$) and 5.1% ± 1.9 ($n = 5$), respectively] (**Figures 2B,C**).

Finally, both IFN-γ after IL-12 + IL-18 stimulation and cytolytic activity of classical NK cells were likewise decreased in CML-CP patients [7.0% ± 2.0 ($n = 8$) and 34.3% ± 11.5 ($n = 5$), respectively] as compared to HD [25.8% ± 3.9 ($n = 15$) and 65.9% ± 8.1 ($n = 6$), respectively] (Figure S2 in Supplementary Material). These findings, together with the similar frequencies of IFN-γ-expressing innate CD8(+) T cells, upon TCR engagement in HD (7.8% ± 6.4; $n = 7$) and CML-CP patients (9.1% ± 2.3; $n = 7$) (Figure S3 in Supplementary Material), would appear to imply that the functional deficiencies of innate CD8(+) T cells in CML-CP patients affect innate rather than adaptive immune responses.

Partial Correction of Quantitative and Functional Deficiencies of Innate CD8(+) T Cells in Patients with CCyR

In patients having achieved complete cytogenetic remission following IM therapy, referred to as CML-IM patients, we observed a recovery of innate CD8(+) T cells (**Figure 3A**, left

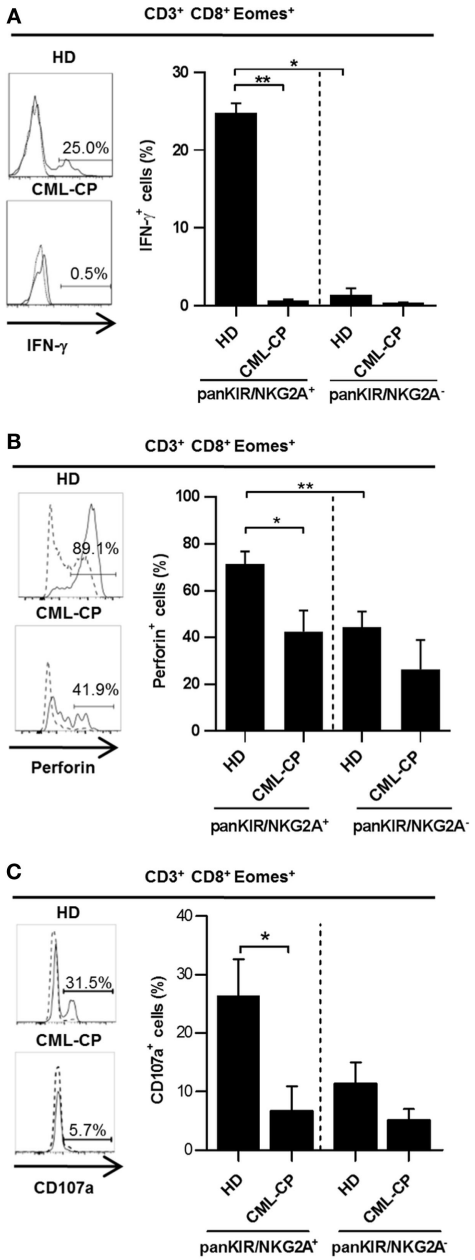

FIGURE 2 | Chronic myeloid leukemia (CML)-CP is associated with acquired functional defects of innate CD 8(+) T cells. Peripheral blood mononuclear cells were cultured and stimulated for 48 h with IL-12 + IL-18 **(A,B)** or IL-15 prior to CD16 triggering **(C)** (see Materials and Methods). IFN-γ **(A)**, perforin **(B)** expression, or CD107a-expressing cells **(C)** (mean ± SEM) were analyzed after gating on killer cell Ig-like receptor (KIR)/NKG2A(+) Eomes(+) CD8(+) CD3(+) cells and KIR/NKG2A(−) Eomes(+) CD8(+) CD3(+) cells in healthy donor (HD) and CML-PC patients. Representative histograms of IFN-γ and perforin expression or CD107a-expressing cells are shown for KIR/NKG2A(+) Eomes(+) CD8(+) CD3(+) cells (filled line) or KIR/NKG2A(−) Eomes(+) CD8(+) CD3(+) cells (dotted line) for each group of HD and CML-CP patients. Without stimulation, frequency of IFN-γ expressing cells was lower than 0.1%. Full cohort data are shown. **(A–C)** Statistical significance to assess differences between populations in HD group or between HD and CML-CP was determined by the two-tailed Wilcoxon or Mann–Whitney non-parametric test, respectively. *$p < 0.05$; **$p < 0.01$.

panel) in CML-IM patients (5.4% ± 0.5; $n = 6$ vs. 2.3% ± 0.6; $n = 7$ in CML-CP patients), back close to the proportions found in HD (7.8% ± 0.9; $n = 16$), while Eomes expression (**Figure 3A**, right panel) was increased in CML-IM patients (MFI: 2.5 ± 0.5; $n = 6$) as compared with CML-CP patients (1.4 ± 0.2; $n = 7$) and was partially restored relative to HD (MFI: 4.0 ± 0.5; $n = 16$). Accordingly, the proportion of cells expressing Eomes in the KIR/NKG2A(+) CD8 T cell subset was restored in CML-IM patients (42.7% ± 2.9; $n = 6$) as compared with CML-CP patients (26.3% ± 3.2; $n = 6$), reverting to the proportions found in HD (46.5% ± 4.6; $n = 14$) (Figure S1 in Supplementary Material).

Importantly, in these conditions, IFN-γ expression in response to IL-12 + IL-18 was partially restored in the innate CD8(+) T cell compartment from CML-IM patients (7.7% ± 2.3; $n = 6$ vs. 0.7% ± 0.1; $n = 5$ in CML-CP patients) relative to HD (24.8% ± 1.2; $n = 6$) (**Figure 3B**), while cytolytic activity, measured as frequencies of CD107a-expressing cells in KIR/NKG2A(+) Eomes(+) CD8(+) CD3(+) cells, returned to normal (27.3% ± 8.3; $n = 6$ vs. 6.8% ± 4.1; $n = 5$ in CML-CP patients), relative to HD (26.4% ± 6.2; $n = 6$) (**Figure 3C**).

Positive Correlation between iNKT Cell PLZF Expression and Innate CD8(+) T Cell Eomes Expression

We have previously reported that IL-4 production by iNKT cells returned to normal in patients having achieved complete CML remission with TKI therapy (22). Given that, in mice, the differentiation of innate CD8(+) T cells depends mainly on PLZF-expressing iNKT cells *via* their IL-4 production (14, 15); we reasoned that the same phenomenon might be applied to humans. In accordance with this notion, we found a significant positive correlation between the levels of Eomes in KIR/NKG2A(+) CD8(+) T cells and of PLZF in iNKT cells including all the HD, CML-CP, and CML-IM samples available (**Figure 4A**). Moreover, we found that after 7 days of culture in the presence of IL-4, recovery of CD8(+) T cells was slightly, but significantly, increased both in terms of frequency and numbers as compared to the total CD3(+) CD8(+) cells (**Figures 4B,C**). We also confirmed in humans that IL-4 strongly enhances Eomes expression both in total CD3(+) CD8(+) cells and in innate CD8(+) T cells (**Figure 4D**). Taken together, these findings support the possible involvement of iNKT cells through their IL-4 production in the generation/maintaining of innate CD8(+) T cells in CML patients.

DISCUSSION

Although the concept of innate memory CD8(+) T cells is now well established in mice, whether an equivalent T-cell population exists in humans remains under debate. We recently reported that CD8(+) T cells co-expressing Eomes and KIR/NKG2A may represent a new, functionally distinct innate T cell subset in humans. Here, by extending our study to CML, we report that this disease is associated with an acquired and reversible defect of innate CD8(+) T cells. To the best of our knowledge,

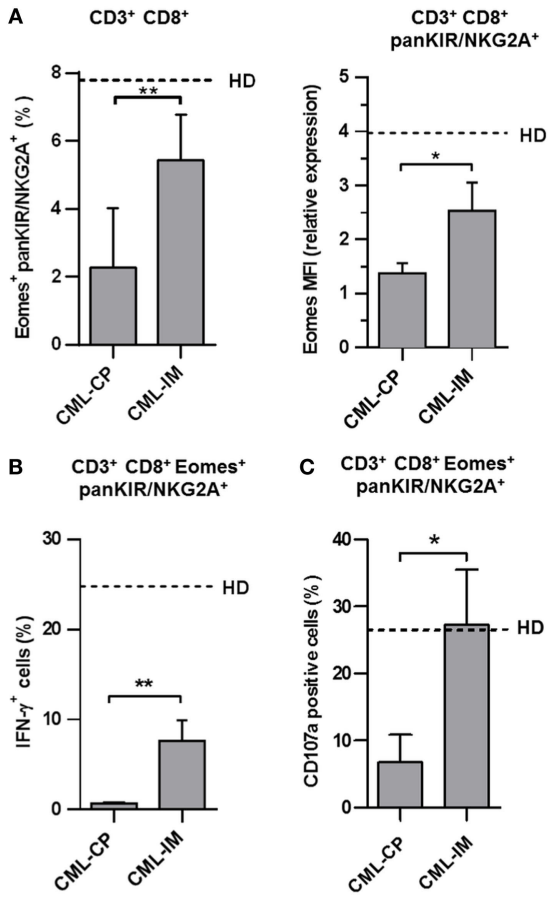

FIGURE 3 | The innate CD8 T cell subset is partially restored in patients having achieved complete cytogenetic remission with imatinib mesylate (IM) therapy. **(A)** Partial restoration of the pool of innate CD8(+) T cells in chronic myeloid leukemia (CML)-IM. Frequency (mean ± SEM) of killer cell Ig-like receptor (KIR)/NKG2A(+) Eomes(+) cells among total CD8(+) CD3(+) cells (left panel) in CML-CP and CML-IM patients. Dotted lines represent mean frequency values in healthy donor (HD). Mean fluorescence intensity (MFI) of Eomes expression (mean ± SEM, right panel) in KIR/NKG2A(+) CD8(+) T cells from CML-CP and CML-IM patients were analyzed after gating on KIR/NKG2A(+) CD8(+) CD3(+) cells. MFI of Eomes expression was normalized on MFI of Eomes from total KIR/NKG2A(–) CD3(–) cells. Dotted lines represent mean relative MFI Eomes values in HD. **(B)** Partial restoration of IL-12 + IL-18-induced IFN-γ expression by innate T CD8(+) cells from CML-IM patients. For details, see the caption of **Figure 2A**. Histograms represent frequencies (means ± SEM) of IFN-γ-expressing cells among KIR/NKG2A(+) Eomes(+) CD8(+) T cells after IL-12 + IL-18 stimulation in each group of patients and HD. Without stimulation, frequency of IFN-γ-expressing cells was lower than 0.1%. **(C)** Complete restoration of natural cytotoxic activity by innate T CD8(+) cells from CML-IM patients. CD107a degranulation/expression after CD16 triggering (for details, see Section "Materials and Methods") in KIR/NKG2A(+) Eomes(+) CD8(+) CD3(+) cells was analyzed by flow cytometry among peripheral blood mononuclear cells preincubated for 48 h with IL-15. Data (means ± SEM) are expressed as frequencies of CD107a-expressing cells in KIR/NKG2A(+) Eomes(+) CD8(+) CD3(+) cells from CML-CP and CML-IM patients. Dotted lines represent mean frequency values in HD. **(A–C)** Statistical significance was determined by the two-tailed Mann–Whitney non-parametric test (comparisons between HD and CML-CP patient groups) or the two-tailed Wilcoxon non-parametric test [comparisons between KIR/NKG2A(+) Eomes(+) CD8(+) CD3(+) cells and KIR/NKG2A(–) Eomes(+) CD8(+) CD3(+) cells from HD or CML-CP patient groups]. *$p < 0.05$; **$p < 0.01$.

these findings are the first providing insights into the potential role of innate CD8(+) T cells in a physiopathological context in humans.

The finding that CML, at diagnosis is closely associated with a profound quantitative and functional deficiency in the innate CD8(+), T cell pool is in line with our hypothesis that this new subset may be involved during tumorigenesis in humans. This assumption was earlier based on our demonstration of terminally differentiated effector features of innate CD8(+) T cells in humans, such as rapid production of IFN-γ and induction of cytolytic function upon stimulation *in vitro*. In the present study, our revelation of the profound impact of CML on these potential antitumoral functions of innate CD8(+) T cells, together with those of NK (see Figure S2 in Supplementary Material) and iNKT cells (22, 27), support a role in cancer immune surveillance of innate CD8(+) T cells analogous to the two other innate cell pools. Even though these findings corroborate the concept that innate CD8(+) T cells, like iNKT cells, may act against tumors in a TCR-independent and NK-like manner, the possibility that these cells recognize leukemia cells cannot be excluded and deserves further investigation.

Remarkably, deficiencies of innate CD8(+) T cells found at diagnosis in CML patients were significantly reversed upon remission following TKI therapy. These data are consistent with the hypothesis that reconstitution of the pool of innate CD8(+) T cells and its partial functional restoration together with those of NK cells and iNKT cells effectively contribute to disease control of CML during TKI therapy. To definitively confirm this assumption, it will be important to further evaluate whether the emergence of innate CD8(+) T cells in terms of both number and functional potential is closely related to long-term remission after treatment discontinuation.

To date, in mice, even though the effector functions of innate/memory CD8(+) T cells have been extensively tested *in vitro*, their actual role during immune responses remains poorly documented. Testing the *in vivo* response following infection with the bacteria *Listeria monocytogenes*, innate/memory CD8(+) T cells were found to have a protective function (5, 6). As far as immune responses against tumors are concerned, only in lymphodepleted mice, does there exist evidence that these cells can enhance response to tumors (30–33). Given our findings, it remains to be directly determined, using experimental tumor models in mice, whether or not innate CD8(+) T cells exert protective functions.

The precise mechanisms for the generation and/or maintenance of innate CD8(+) T cells in humans remain to be determined. In the mouse, a key role in Eomes upregulation has been attributed to PLZF-expressing T cells. Similarly, our data in peripheral blood show a close correlation between the levels of Eomes in innate CD8(+) T cells and of PLZF in iNKT cells. Moreover, expression of PLZF and Eomes in iNKT cells and innate CD8(+) T cells, respectively, were both found to be reduced in patients at CML diagnosis, and reversed after disease remission, as were innate CD8(+) T cells in terms of both number and functions. Taken together, these findings are consistent with the existence of a PLZF-dependent mechanism in humans, similar to that reported in mice. It remains to

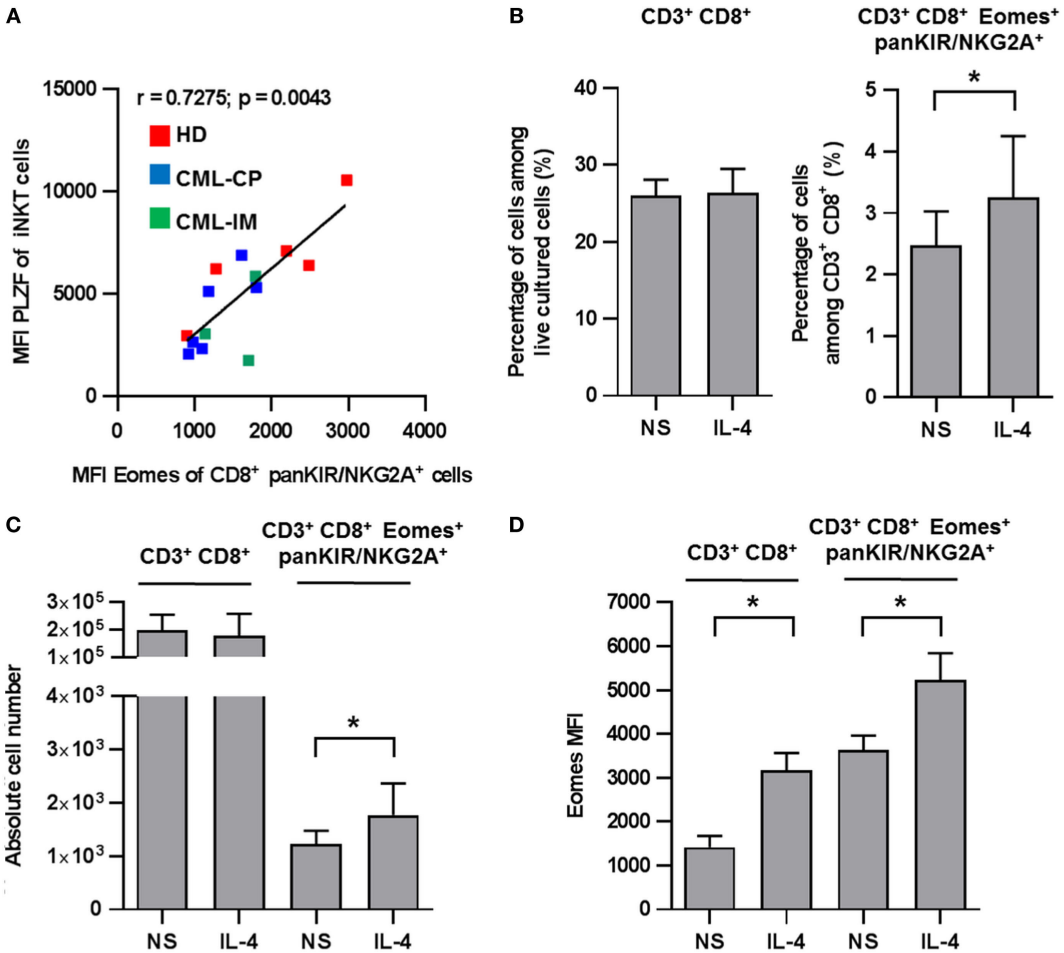

FIGURE 4 | **(A)** Positive correlation between invariant natural killer T (iNKT) cell promyelocytic leukemia zinc finger (PLZF) expression and innate CD8 T cell Eomes expression. Eomes and PLZF expression were analyzed in innate CD8(+) T cells and iNKT cells, respectively, among peripheral blood mononuclear cells (PBMCs) by flow cytometry *ex vivo* after cellular permeabilization. Eomes expression and PLZF were analyzed after gating on killer cell Ig-like receptor (KIR)/NKG2A(+) CD8(+) CD3(+) cells and 6B11(+) CD3(+) cells, respectively. Mean fluorescence intensity (MFI) values are expressed relative to that of isotype control. Data from healthy donor ($n = 5$), chronic myeloid leukemia (CML)-CP ($n = 6$), and CML-imatinib mesylate ($n = 3$) were pooled. The MFI of PLZF-expressing iNKT [6B11(+) CD3(+)] cells correlate positively with MFI of Eomes expression on innate CD8(+) T [KIR/NKG2A(+) Eomes(+) CD8(+) CD3(+)] cells (correlation Spearman test; $n = 14$, $r = 0.7275$, $p = 0.0043$). **(B–D)** Innate CD8(+) T cells depend on IL-4 for homeostasis/expansion. PBMCs from five HDs were cultured for 7 days with IL-4 or medium alone (not stimulated, NS) and then analyzed by flow cytometry *ex vivo* after cellular permeabilization for innate CD8(+) T cells. Frequency **(B)**, absolute cell number **(C)**, and Eomes MFI **(D)** (mean ± SEM) of CD3(+) CD8(+) cells among total live PBMCs (left) and KIR/NKG2A(+) Eomes(+) CD8(+) CD3(+) cells among CD3(+) CD8(+) cells (right) are shown. Statistical significance was determined by the one-tailed Wilcoxon non-parametric test. $^*p < 0.05$; $^{**}p < 0.01$.

determine whether, as in mice, PLZF-expressing T cells, especially iNKT cells, promote the development of blood peripheral innate T CD8(+) T cells by providing IL-4. In accordance with this view, we recently demonstrated a functional deficiency of IL-4 production by iNKT cells in CML patients at diagnosis and its partial restoration in patients having achieved remission after TKI therapy (22).

Further investigations are required to elucidate the exact mechanisms accounting for the deficiency of innate CD8(+) T cells at diagnosis and the beneficial effect of TKI therapy on this pool of cells. Nonetheless, from our data, it is tempting to speculate that the dysfunctions of innate CD8(+) T cells in CML patients at diagnosis that are remedied by TKI therapy originate from antigen APC-dependent dysfunctions, which in turn might contribute to iNKT cell deficiencies. This hypothesis arises from our recent findings showing that BCR-ABL from CML myeloid DCs mediates iNKT-cell immune subversion by downregulating cell-surface CD1d expression (27). Another non-exclusive mechanism may involve IL-15, which is required for maintenance not only of conventional memory CD8(+) T cells (34, 35) but also innate/memory CD8(+) T cells (32, 36). Indeed, CD8α(+) DC trans-presentation of IL-15 contributes to development of innate-like CD8(+) T cells in the periphery (37). For these reasons, the possible existence of a deficit in IL-15 expression and/or in IL-15

trans-presentation by mDCs from CML-CP patients is deserving of special attention.

All in all, by revealing an iNKT/innate CD8(+) T-cell axis with expected physiopathological relevance in CML, our findings should enhance understanding of the T cell components restored in CML treatment that contribute to disease control. From a more general standpoint, our study underscores the potential role of innate CD8(+) T cells against tumors in conjunction with iNKT cells and may contribute significantly to our understanding of the role of these innate T cell subsets in the development of protective immunity in humans.

AUTHOR CONTRIBUTIONS

FJ, EC, and AB designed the experiments, performed the experiments, analyzed and interpreted the data, and wrote the manuscript. DD, AL, and SB designed the experiments, performed the experiments, and analyzed and interpreted the data. AR, NP, and LL provided assistance with cell cultures. CG contributed to PBMC preparation from patients and healthy controls. FG and LR provided clinical samples and contributed to the interpretation of data. AH and J-MG together were responsible for the overall study design, supervised the project, and took primary responsibility for writing the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2016.00688/full#supplementary-material.

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Phenotype of NK-Like CD8(+) T Cells with Innate Features in Humans and Their Relevance in Cancer Diseases

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Unconventional T cells are defined by their capacity to respond to signals other than the well-known complex of peptides and major histocompatibility complex proteins. Among the burgeoning family of unconventional T cells, innate-like CD8(+) T cells in the mouse were discovered in the early 2000s. This subset of CD8(+) T cells bears a memory phenotype without having encountered a foreign antigen and can respond to innate-like IL-12 + IL-18 stimulation. Although the concept of innate memory CD8(+) T cells is now well established in mice, whether an equivalent memory NK-like T-cell population exists in humans remains under debate. We recently reported that CD8(+) T cells responding to innate-like IL-12 + IL-18 stimulation and co-expressing the transcription factor Eomesodermin (Eomes) and KIR/NKG2A membrane receptors with a memory/EMRA phenotype may represent a new, functionally distinct innate T cell subset in humans. In this review, after a summary on the known innate CD8(+) T-cell features in the mouse, we propose Eomes together with KIR/NKG2A and CD49d as a signature to standardize the identification of this innate CD8(+) T-cell subset in humans. Next, we discuss IL-4 and IL-15 involvement in the generation of innate CD8(+) T cells and particularly its possible dependency on the promyelocytic leukemia zinc-finger factor expressing iNKT cells, an innate T cell subset well documented for its susceptibility to tumor immune subversion. After that, focusing on cancer diseases, we provide new insights into the potential role of these innate CD8(+) T cells in a physiopathological context in humans. Based on empirical data obtained in cases of chronic myeloid leukemia, a myeloproliferative syndrome controlled by the immune system, and in solid tumors, we observe both the possible contribution of innate CD8(+) T cells to cancer disease control and their susceptibility to tumor immune subversion. Finally, we note that during tumor progression, innate CD8(+) T lymphocytes could be controlled by immune checkpoints. This study significantly contributes to understanding of the role of NK-like CD8(+) T cells and raises the question of the possible involvement of an iNKT/innate CD8(+) T cell axis in cancer.

Keywords: innate memory CD8(+) T cells, NK-like T cells, iNKT cells, natural killer receptors, Eomesodermin, CD49d, chronic myeloid leukemia, solid cancers

INTRODUCTION

The traditional view of the immune system distinguishes innate immunity from adaptive or acquired immunity. Innate immunity is derived from cells expressing the receptors specific for molecules from microbial pathogens called pathogen-associated molecular patterns or self-molecules from the healthy or unhealthy individual called damage-associated molecular patterns. One of the key characteristics of innate immunity effector mechanisms is their capacity for very rapid response to pro-inflammatory cytokines such as IL-12, IL-18, and IL-33.

The effectors of adaptive immunity possess receptors characterized by highly diverse and specific antigens. A major feature of adaptive immunity consists in its serving as an essential support for the immunologic memory, which means that it can remember and quite effectively respond to an antigen long after having encountered it for the first time.

However, numerous works over the past 20 years have shown the distinction between innate immunity and adaptive immunity to be less clear-cut and more tenuous than it first appeared. This revised perception is based, in particular, on description of non-conventional T cells responding to *stimuli* that had previously been considered as being recognized solely by innate cells. These populations of T lymphocytes include not only certain T-cell receptor (TCR)-γδ cells but also TCR-αβ cells such as natural killer T (iNKT) cells and innate mucosal-associated invariant T (MAIT) cells [for a list of the different cells, see Ref. (1, 2)].

A new contingent of innate T cells was described in the early 2000s in the mouse, partially in the thymus, where they were termed «innate memory» (IM) CD8(+) T cells, and partially in the spleen, where they were termed «virtual memory» (VM) CD8(+) T cells (3, 4). Aside from possessing a phenotype of activated memory cells, one characteristic of these cells consists in their differentiating into memory cells independently of a foreign antigen. In parallel, CD8(+) T cells in humans were described as cells possessing innate characteristics including NK markers. They were found in human cord blood, a finding consistent with the hypothesis that their development does not depend on foreign antigens. These cells hence were termed NK-like CD8(+) T cells.

At the outset of this review, we shall compare the human NK-like CD8(+) T cells with IM/VM CD8(+) T cells in mice. On the basis of this comparison and with regard to humans, we shall focus first on expression of the transcription factor Eomesodermin (Eomes) as a lineage marker of that population of cells, and then on their innate functions (cytotoxicity and TCR-independent IFN-γ expression), along with their memory phenotype, and on the roles of IL-4- and promyelocytic leukemia zinc-finger factor (PLZF)-expressing T cells in differentiation of these cells, hereafter referred to as innate CD8(+) T cells. We shall discuss the use of membrane markers, particularly the α4-integrin CD49d, in order to obtain a more well-defined phenotype correlating with their functions and/or explaining their possible physiological role. Finally, we shall discuss the implication of innate CD8(+) T cells in anticancer immunity in humans.

INNATE CD8(+) T LYMPHOCYTES IN MICE

Studies conducted shortly after 2000 by Forman et al. (5, 6) demonstrated the existence of CD8(+) T cells producing IFN-γ in response to innate signals occurring independently from the TCR. These CD8(+) T cells possessed a CD44(+) CD62L(−) CD122(+) memory phenotype and, *in vivo*, provided protection against *Listeria monocytogenes* (LM) infection (5–7). Their mobilization depended on the production of IL-12 and IL-18. Interestingly, the Forman team subsequently showed that this cell population was present in the thymus of C57BL/6 wild-type mice and that it was enriched in C57BL/6 H-2 $K^{b-/-}D^{b-/-}$ mice not having undergone stimulation by foreign antigens (7).

A second series of studies having to do with $Itk^{-/-}$ (inducing T cell kinase), $Rlk^{-/-}$ (resting lymphocyte kinase), or $Itk^{-/-}Rlk^{-/-}$ mice led to identification of a population of thymic CD8(+) T cells expressing an activated memory [CD44(+) CD62L(−) CD122(+)] phenotype and called IM CD8(+) T cells (8–12). Interestingly, these cells developed in the thymus and are exported to the spleen and the lymph nodes (LNs) where they could fulfill an anti-infective function against LM, particularly through production of IFN-γ following TCR-independent stimulation by IL-12 and IL-18. An important point in the studies dedicated to this population has been the demonstration that its differentiation depended on expression of the Eomes transcription factor and IL-15 (13–15). Eomes expression initiates the differentiation program of these cells and induces the expression of CD122 (the β chain of the IL-2 and IL-15 receptor). IL-15 has a critical role in the expansion of IM CD8(+) T cells (8, 16). It should also be noted that this population is present in $Itk^{-/-}K^{b-/-}D^{b-/-}$ mice, a finding suggesting that at least some IM CD8(+) T cells are selected by non-classical major histocompatibility complex (MHC) class I molecules (9, 17–19).

A final series of studies has described in mouse spleens and peripheral lymphoid organs a population of activated [CD122(+)] and memory [CD44(+) CD62L(−)] CD8(+) T lymphocytes of which the differentiation into memory cells occurs independently of any recognition of a foreign antigen. This population consists in the so-called VM CD8(+) T cells. As is the case with IM T CD8(+) cells, Eomes and IL-15 are the two key factors in their differentiation. This population is capable of producing IFN-γ in response to innate stimulation by IL-12 + IL-18 (11, 20–22).

The expression by mouse thymic IM CD8(+) T cells of some integrins, such as CD49d [an α4-integrin, or VLA-α4, which is most often matched with a β7-integrin (which is bound to Madcam and VCAM-1 or CD106), or a β1-integrin], has been described (14, 21, 23, 24). Hence, CD49d is used to discriminate between IM and VM T cells (**Figure 1A**) arising from the thymus or the spleen, respectively. In this model, a possible filiation link between IM CD8(+) and VM CD8(+) T-cell populations remains to be investigated. A gating strategy to identify IM/VM CD8(+) T cells in mice, taking Eomes, CD44, and CD122 together with CD49d as delineating markers is depicted in **Figure 1B** and Figure S1 in Supplementary Material.

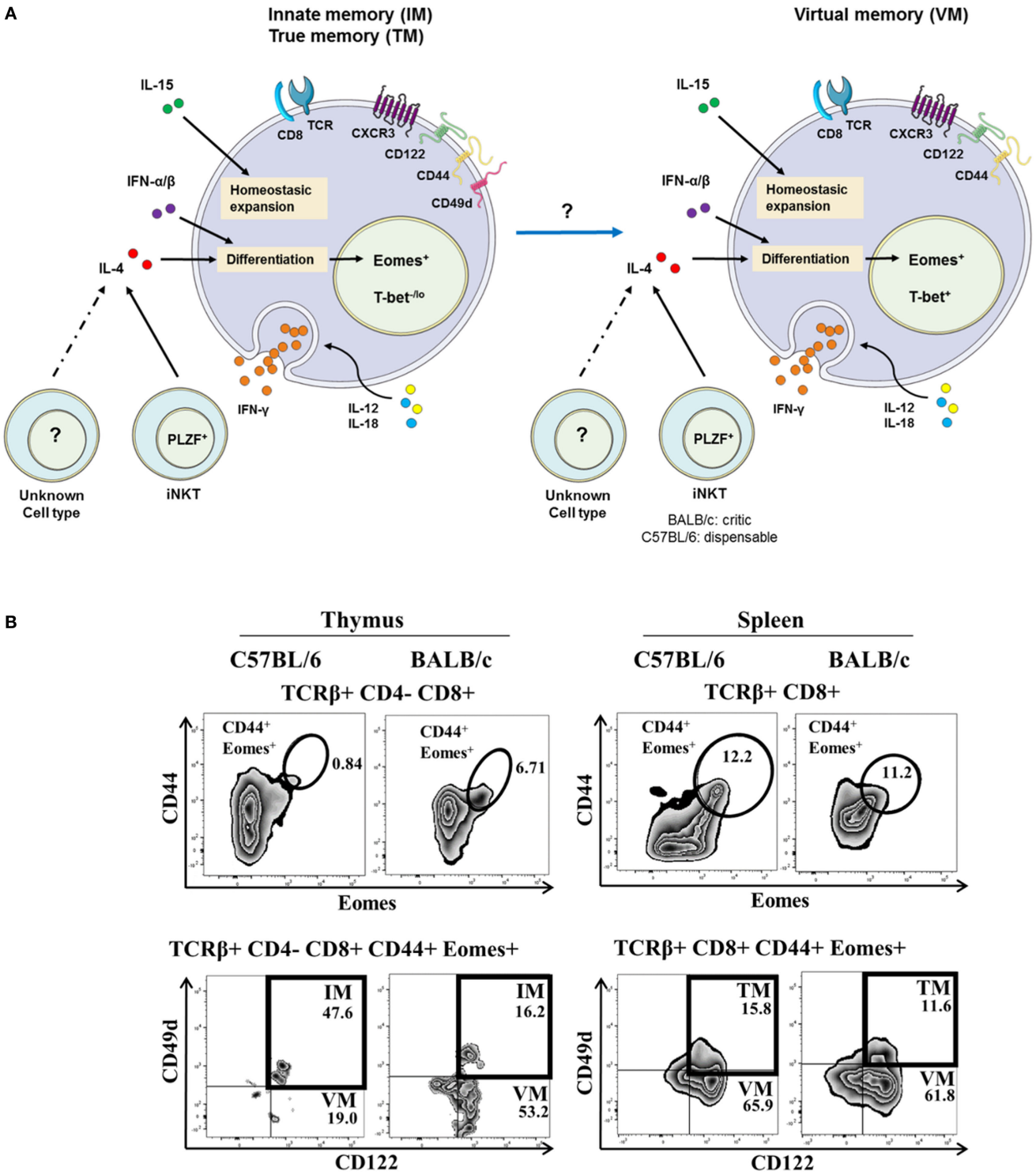


FIGURE 1 | **The innate/true memory (IM/TM) and virtual memory (VM) CD8(+) T cells in mice**. **(A)** Representative view of IM/TM and VM CD8(+) T cells in mice. CXCR3(+) CD122(+) CD44(+) CD49d(+) Eomes(+) and CXCR3(+) CD122(+) CD44(+) Eomes(+) T-bet(+) expression defined the IM/TM CD8(+) and the VM CD8(+) T-cell subsets, respectively. Both subsets have the capacity to respond to the IL-12 + IL-18 innate-like stimulation, and their development and homeostasis are regulated by IL-4 [mainly produced by PLZF(+) iNKT cells], IL-15, and type I interferons. Note that possible existence of a filiation link in mice between IM CD8(+) and VM CD8(+) T-cell populations remains to be investigated. **(B)** Gating strategy in C57BL/6 and BALB/c mouse strains for IM and VM CD8(+) T cells in the thymus (left panel) and for TM and VM CD8(+) T cells in the spleen (right panel). IM/TM and VM population frequencies are displayed in the upper and lower right quadrants, respectively.

Based on this gating strategy, in the C57BL/6 mouse strain, the vast majority of memory [CD44(+) Eomes(+) CD122(+)] CD8(+) T cells are IM CD8(+) T cells in the thymus vs. VM CD8(+) T cells in the spleen, as attested by their differential CD49d expression. Interestingly, in the BALB/c strain, thymic IM CD8(+) T cells are minority cells among memory CD44(+) CD122(+) Eomes(+) cells, raising the question of a possible link between IM and VM CD8(+) T cells, as well as the association of CD49d with the innate functions of IM/VM CD8(+) T cells (see CD49d, a Functional Marker of Innate CD8(+) T Cells in Humans).

IL-15 plays a key role in the homeostatic expansion of CD8(+) T cells, and it has been reported in several studies that this cytokine is implicated in differentiation into VM CD8(+) T cells after homeostatic proliferation (4, 25–28). However, August and his team have shown that the size of the IM/VM CD8(+) T population is not modified by T-cell depletion prior to bone marrow transplantation, a finding suggesting that lymphoid precursors are differentiated into IM/VM CD8(+) T cells following "tuning" by cells expressing MHC class I molecules. Moreover, in the same study, IM/VM CD8(+) T cells are distinguished from homeostatic proliferation CD8(+) T cells by a different transcriptional profile (29). Another study suggests that contrary to naive CD8(+) T cells and homeostatic proliferation CD8(+) T cells, acquisition of the phenotype of IM CD8(+) T cells necessitates engagement of their TCR (30).

EVIDENCE FOR NK-LIKE CD8(+) T CELLS IN HUMANS

In parallel with the previously described work on mice, several studies conducted in the early 2000s demonstrated the existence in humans of CD8(+) T cell-expressing markers and receptors of NK cells including CD56, KIR, NKG2A and NKG2C (CD159a and c), and CD94 (31–36). Several studies precisely characterized the phenotype of the KIR(+) CD8(+) T cells and showed that they possess an EMRA memory phenotype [CD45RA(+) CCR7(−) CD57(+)] (33, 34). Finally, Björkström et al. (33), showed that EMRA(+) KIR(+) CD8(+) T cells have a skewed repertoire using fewer different Vβ than their EMRA(+) KIR(−) CD8(+) T cell counterpart, a finding suggesting the role of antigenic pressure in the acquisition of this phenotype. An equivalent to this population of KIR/NKG2(+) CD8(+) T cells is present in human cord blood, where they possess an EMRA memory phenotype and rapidly express IFN-γ following TCR stimulation (36). In fact, this is a population of T cells that has been educated and has differentiated in the absence of foreign antigenic stimulation, into terminal effector memory T cells.

These KIR(+) CD8(+) T cells have a weaker response to TCR stimulation than their KIR(−) CD8(+) counterparts with regard to the expression of IFN-γ and TNF-α or to the degranulation evaluated by CD107a staining. The KIR(+) CD8(+) T cells expressing two different KIRs have a weaker response to TCR stimulation than cells expressing a single KIR or without KIR (33, 34). Remarkably, this CD56(+) [or KIR(+)] CD8(+) T cell subset responds quite effectively to innate *stimuli*, one example being the association of IL-12 with IL-18 (37). The same team reported that loss of this function in *IL-12R*$^{-/-}$ patients is associated with a risk of severe infections from intracellular germs, particularly mycobacteria and *Salmonella* (37).

HUMAN NK-LIKE CD8(+) T CELLS EXPRESS EOMES AND DISPLAY INNATE FUNCTIONS

The study by Jacomet et al. (38) showed that KIR(+) and/or NKG2A(+) CD8(+) T cells preferentially express Eomes. These KIR/NKG2A(+) Eomes(+) CD8(+) T cells have a memory phenotype and share functional and phenotypic features (4, 38) with IM CD8(+) T cells in mice (8–11). Moreover, with respect to KIR/NKG2A(−) CD8(+) T cells, they possess an EMRA phenotype [CD45RA(+) CCR7(−)] and preferentially express the surface molecule CD57, which is a terminal differentiation marker (**Figure 2A**). **Figure 2B** shows a gating strategy designed to analyze these cells in human blood. Remarkably, the majority (around 60–70%) of KIR/NKG2A(+) Eomes(+) CD8(+) T cells express the T-box transcription factor T-bet, a phenotype comparable to the phenotype described for mouse VM CD8(+) T cells (22). These cells are characterized by increased frequency of CD16(+) cells in comparison to conventional memory [KIR/NKG2A(−) Eomes(+)] CD8(+) T cells. Moreover, CD16 expression is substantially increased in KIR/NKG2A(+) Eomes(+) CD8(+) T cells following 48 h of IL-15 *in vitro* treatment (**Figure 3A**).

Together with their marked NK-like phenotype, a hallmark of KIR/NKG2A(+) Eomes(+) CD8(+) T cells is their capacity to rapidly produce large amounts of IFN-γ in response to innate-like stimulation by IL-12 + IL-18 (38). So it is that among the CD8(+) T cells, 60–70% of those possessing a capacity for innate response to IL-12 + IL-18 are KIR/NKG2A(+) Eomes(+) CD8(+) T cells. Moreover, the frequency of the cells producing IFN-γ in response to IL-12 + IL-18 stimulation is four times greater than that of the same cells stimulated with anti-CD3 and anti-CD28 agonistic monoclonal antibodies (mAbs) (38). In the same way, these cells possess a cytotoxic arsenal: perforin and granzyme B. They possess an innate cytotoxic potential induced by an anti-CD16 antibody and revealed by the CD107a test, which assesses degranulation (38). In the final analysis, these data demonstrate that KIR/NKG2A(+) Eomes(+) CD8(+) T cells display innate activity by responding to innate *stimuli* with response efficacy greater than that of their response to adaptive *stimuli* (*i.e.*, *via* their TCR), as has also been shown for innate T-cell populations such as dendritic epidermal T cells (39).

A final element appearing to favor the innate character of KIR/NKG2A(+) Eomes(+) CD8(+) T cells is their differentiation without any exogenous antigenic stimulation, as it is possible to show the existence of these cells in the fetal thymus (40), and as our team has shown them to be present in cord blood. Interestingly, in cord blood, they express an EMRA phenotype with a lower CD57 cell frequency than in the adult, suggesting that there exist supplementary steps in the terminal maturation of these peripheral cells (38). Functionally, as is the

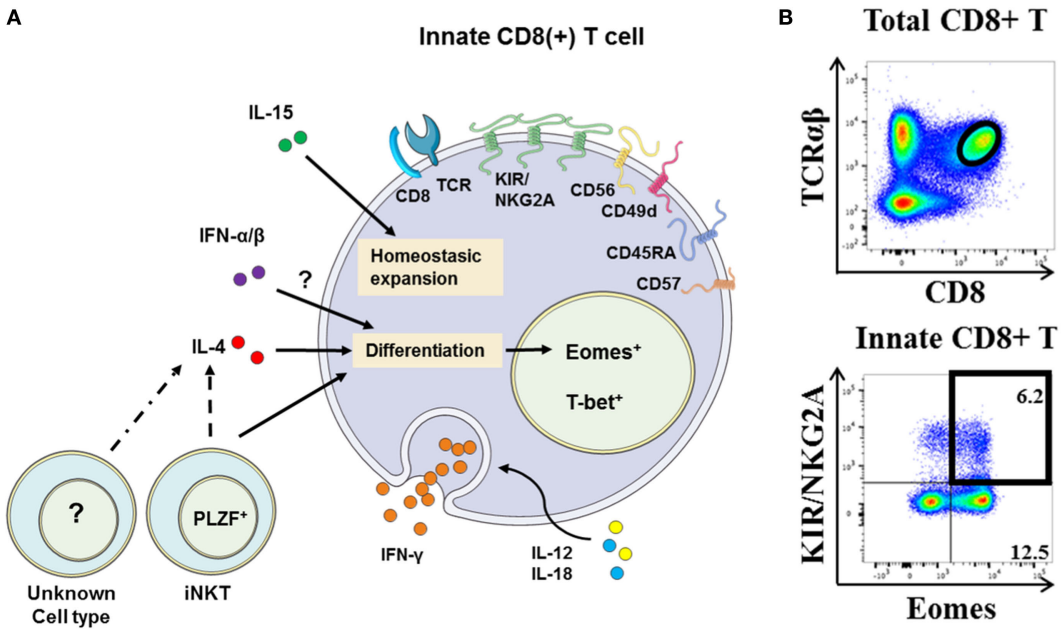


FIGURE 2 | **The innate CD8(+) T cells in humans**. **(A)** Representative view of human innate CD8(+) T cells. The membrane markers KIR/NKG2A, CD56, CD57, CD45RA, and CD49d and nuclear transcription factors Eomes and T-bet define this new unique innate CD8(+) T-cell population [for details, see Ref. (4, 38)]. Innate CD8(+) T cells in humans have the capacity to respond to IL-12 + IL-18 innate-like stimulation. Their development and homeostasis are regulated by IL-15, IL-4 [presumably by PLZF(+) iNKT cells], and expectedly type I interferons. Note that the precise origin (central vs. peripheral) of innate CD8(+) T-cell populations in humans remains under debate. **(B)** Gating strategy for innate CD8(+) T cells among peripheral blood mononuclear cells from healthy donors. After gating on T-cell receptor (TCR)-αβ(+) CD8(+) cells, the innate CD8(+) T cells are defined as KIR/NKG2A(+) Eomes(+). Frequency of innate CD8(+) T cells is displayed in the upper right corner.

case in adult cells, these KIR/NKG2A(+) Eomes(+) CD8(+) T cord blood cells express IFN-γ after innate-like stimulation by IL-12 + IL-18.

All in all, we described for the first time a CD8(+) T cells population with innate features by associating Eomes and KIR/NKG2A markers with the capacity to respond to IL-12 + IL-18 stimulation. These features validate the KIR/NKG2A(+) Eomes(+) CD8(+) T-cell compartment as a new member of the innate T cell family in humans, and we have termed them innate CD8(+) T cells (41).

There exist a number of hypotheses on the mechanisms involved in the reprogramming of conventional T lymphocytes into innate CD8(+) T lymphocytes. One of these assumptions is based on a cross talk between IL-12R and TCR signalosome, in which IL-12 recruits Tyk2 and Fyn tyrosine kinases to activate CD3ζ–TCR signal transduction pathways (42).

Some studies conducted in mice suggest that IM and/or VM CD8(+) T cells could be selected by non-classical MHC class I molecules (9, 17–19). In humans, our results (Figure S2 in Supplementary Material) show that a relatively small fraction of KIR/NKG2A(+) Eomes(+) CD8(+) cells are MAIT cells. This finding suggests that at least some human innate CD8(+) T cells could be selected by non-classical class I MHC molecules, and it raises the possibility of the presence in this population of cells being restricted by non-classical MHC class I HLA-E molecules (18).

CD49d, A FUNCTIONAL MARKER OF INNATE CD8(+) T CELLS IN HUMANS

We have sought out other markers of innate CD8(+) T cells in humans. Among them, we tested CD56, a marker in humans associated with NK cells, but our results showed that this marker is no more effective in distinguishing the innate CD8(+) T-cell population than the KIR/NKG2A markers. More precisely, this marker delineate only 20–30% of the CD8(+) T cells expressing IFN-γ after innate stimulation by IL-12 + IL-18 (as opposed to the approximately 70% exhibited by the KIR/NKG2A markers) [data not shown; (38)]. We also tested CD161, of which the expression is a common feature of human innate T cell subsets including iNKT cells, TCR-γδ T cells, and MAIT cells (43). However, as for CD56, CD161 is expressed by only approximately 20% of the Eomes(+) KIR/NKG2A(+) CD8(+) T cells (**Figure 3B**).

Reasoning by analogy with the mouse model, we tested CD49d expression by innate CD8(+) T cells in humans (**Figures 4A,B**). Interestingly, the CD8(+) T cells with the KIR/NKG2A(+) Eomes(+) phenotype strongly expressed CD49d, as compared to the CD8(+) T-cell population taken as a whole (**Figure 4A**). Moreover, the majority (almost 70%) of the cells with a more pronounced expression of CD49d consisted in those expressing IFN-γ after innate-like stimulation by IL-12 + IL-18 (**Figure 4B**). These results show CD49d to be closely associated

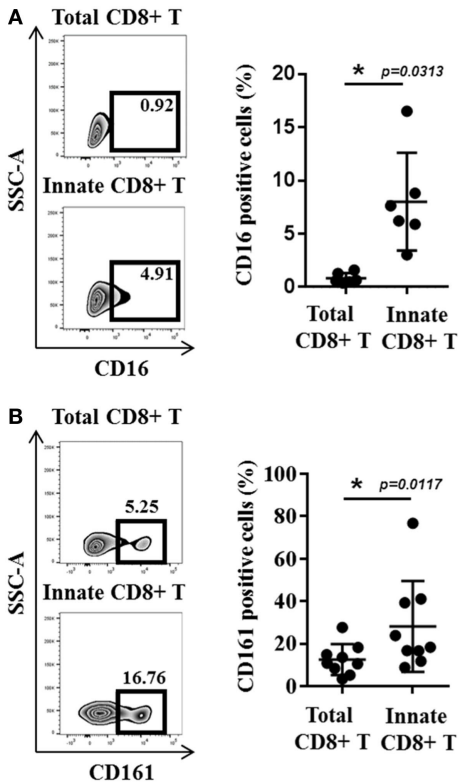


FIGURE 3 | CD16 and CD161 are slightly enriched in innate CD8(+) T cells. **(A)** Peripheral blood mononuclear cells (PBMCs) from healthy donors (HDs) (n = 6) were cultured for 48 h in the presence of IL-15 and then analyzed by flow cytometry. One representative sample is shown for CD16 expression among total (upper panel) and innate (lower panel) CD8(+) T-cell subsets. Frequencies are displayed in the gate and the histogram (right panel) presents the full cohort of CD16-positive cells (mean ± SD) in both T-cell subsets. **(B)** PBMCs from HD (n = 9) were analyzed *ex vivo* by flow cytometry. One representative sample is shown for CD161 expression among total (upper panel) and innate (lower panel) CD8(+) T-cell subsets. Frequencies are displayed in the gates (left panel). The histogram (right panel) represents the full cohort of CD161-positive cells (mean ± SD) in both T-cell subsets.

with the innate effector functions of the innate CD8(+) T cells. On the other hand, CD49d cannot substitute for Eomes and KIR/NKG2A, given the fact that only 20–40% of CD49d(+) cells are actually innate CD8(+) T cells (Figure S3 in Supplementary Material). However, as innate CD8(+) T cells arise from the thymus, the functional link between higher CD49d expression and IFN-γ secretion in response to IL-12 + IL-18 stimulation should be tested in cord blood, which is an accessible source of cells providing an approximate reflection of thymic cells in humans.

These results have led us to inquire about the biological meaning of CD49d expression by thymic vs. splenic (or IM vs. VM) CD8(+) T lymphocytes in mice. The results of several teams (14, 21) suggest that contrary to thymic IM CD8(+) cells, splenic (so-called VM) innate CD8(+) T cells do not express CD49d (14, 21) (**Figure 1**). We have observed, as in humans, that a large majority of splenic CD8(+) T cells expressing IFN-γ after innate-like stimulation by IL-12 + IL-18 were those harboring the IM [Eomes(+) CD44(+) CD122(+)] phenotype (**Figure 4C**). Moreover, these IFN-γ-producing cells were mostly those that more pronouncedly expressed CD49d as compared to the total CD8(+) T-cell population (**Figure 4D**).

Taken as a whole, these different results suggest that CD49d is associated with the innate-like functions of NK-like CD8(+) T cells as much in humans as in mice. According to the β chain with which CD49d is matched, the cells are variably liable to migrate toward different territories. While the α4β7-integrin is associated with the migration of T lymphocytes toward the digestive mucosa, the α4β1-integrin is associated with migration toward the oral mucosa, the salivary glands, the vaginal mucosa, and the central nervous system (44, 45). Indeed, therapeutic targeting of CD49d is used in treatment of multiple sclerosis (MS). Several studies have documented the implication of CD49d in the penetration of pathogenic T lymphocytes during MS or experimental allergic encephalomyelitis (EAE) (46, 47). Taken together, these different observations raise the possibility of the implication of the innate CD8(+) T cells in the pathogenesis of MS/EAE.

THE FACTORS ASSOCIATED/IMPLICATED IN CONTROL OF THE DIFFERENTIATION OF INNATE CD8(+) T LYMPHOCYTES

In mice, differentiation of IM CD8(+) T lymphocytes depends on soluble factors such as type I IFN and the IL-4 and IL-15 cytokines.

Several studies have shown that IL-4 favors the arising of VM CD8(+) T cells (48–51). More recent studies confirm the involvement of IL-4 in IM/VM CD8(+) T cell generation with a more critical role being assumed in the BALB/c (22, 30) than in the C57BL/6 mouse strain (14). IL-4-producing PLZF(+) T cells, including at least partially iNKT lymphocytes (52, 53), elicit the generation of IM CD8(+) T cells. Other results from the Hogquist group confirm the link between iNKT PLZF(+) cells, IL-4, and IM CD8(+) T cell generation by comparing different mouse strains (22). **Figure 1B** shows the higher frequency of T CD8(+) IM in the thymus of BALB/c in comparison with C57BL/6 mice. However, the August group shows results suggesting that IL-4 produced by differentiating CD4(+) CD8(+) double-positive thymocytes controls the generation of IM CD8(+) thymocytes in the $Itk^{-/-}$ C57BL/6 background (16, 29). As concerns VM CD8(+) T lymphocytes, IL-4 is likewise at least partially implicated. IL-4 is likely to act by eliciting Eomes expression, of which the action would entail a heightened level of expression of CD122, and thereby sensitize the cells to IL-15 (14, 16).

Type I IFNs such as IFN-β and/or IFN-α (24) could favor the differentiation of lymphocytes to VM and/or IM CD8(+) T lymphocytes. The underlying mechanism described by the authors is the induction of Eomes expression by naive CD8(+) T cells.

Finally, IL-15 is a determining factor in the maintenance and/or homeostatic expansion of IM and VM CD8(+) T cells (8, 14, 16).

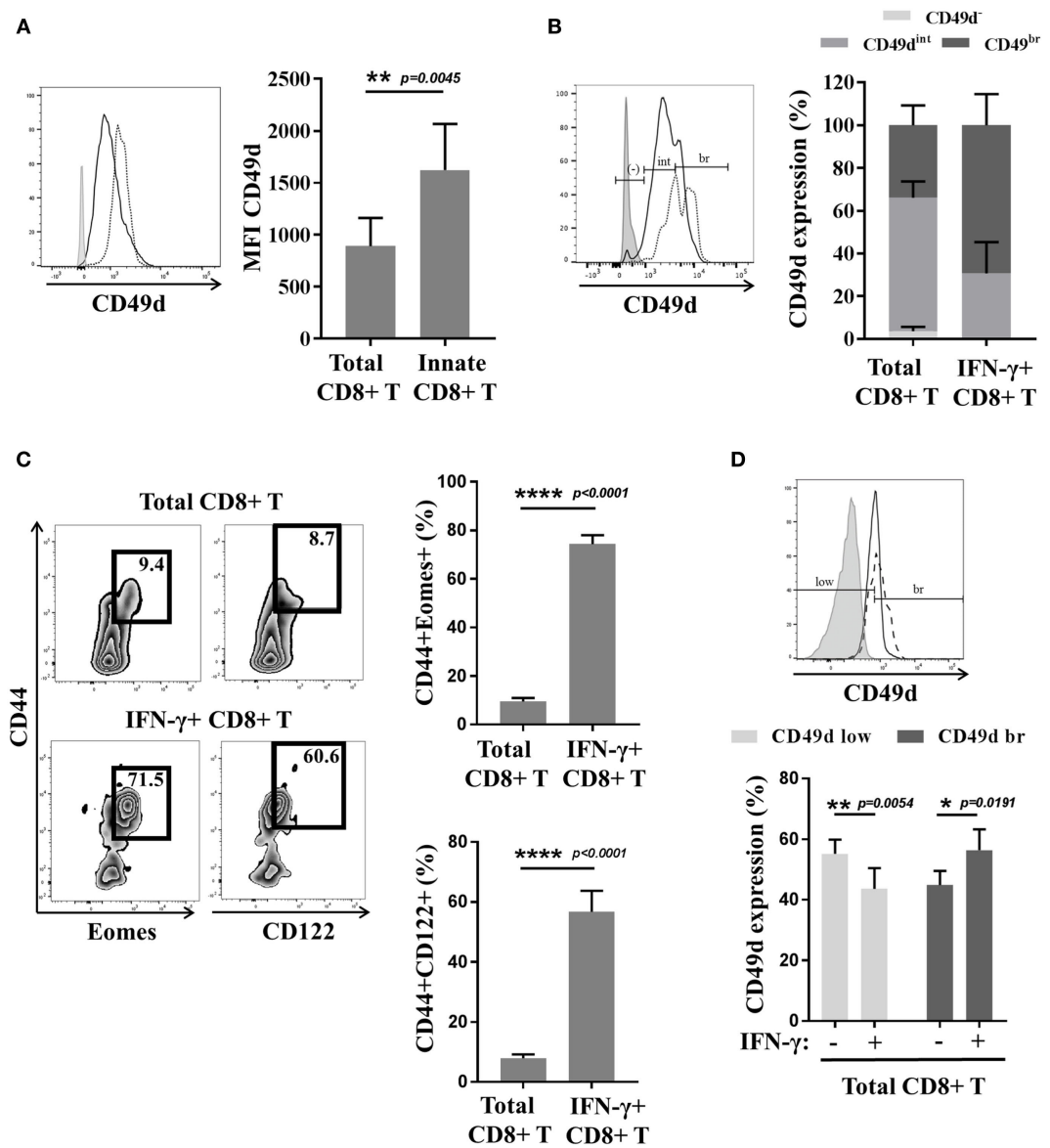


FIGURE 4 | **The CD49d molecule correlates with the innate function of innate CD8(+) T cells in mice and humans**. **(A)** Peripheral blood mononuclear cells (PBMCs) from healthy donors (HDs) ($n = 6$) were analyzed *ex vivo* by flow cytometry for CD49d expression in total and innate CD8(+) T cells. One representative sample is shown (left): total CD8(+) T cells (solid dark line), innate CD8(+) T cells (dotted black line), and isotype control (gray). Full cohort (MFI ± SD) is shown on the histogram (right). **(B)** PBMCs from HD ($n = 5$) were cultured 48 h in the presence of IL-12 + IL-18 and then analyzed *ex vivo* by flow cytometry for CD49d expression. Distribution of CD49d (percentage ± SD) in total CD8(+) T cells and in IFN-γ(+) CD8(+) T cells is shown on the histogram (right). One representative sample is shown (left): total CD8(+) T cells (solid dark line), IFN-γ(+) CD8(+) T cells (dotted black line), and isotype control (gray). **(C,D)** Splenocytes from Eomes-GFP mice were isolated, cultured for 16 h in the presence of IL-12 + IL-18, and analyzed by flow cytometry. **(C)** One representative sample is shown for the presence of CD44(+) Eomes(+) and CD44(+) CD122(+) cells among total CD8(+) (upper left panel) or IFN-γ(+) CD8(+) T cells (lower left panel). Frequency of each subset is displayed in the gate. Full cohort frequencies for CD44(+) Eomes(+) (upper histogram) and CD44(+) CD122(+) (lower histogram) cells among total CD8(+) and IFN-γ(+) CD8(+) T cells are shown. **(D)** CD49d expression was analyzed in total and IFN-γ(+) CD8(+) T cells. One representative sample is shown (upper panel): total CD8(+) T cells (solid dark line), IFN-γ(+) CD8(+) T cells (dotted black line), and isotype control (gray). Full cohort histograms for frequencies of CD49d(low) and CD49d(bright) are shown (lower panel) in total CD8(+) and IFN-γ(+) CD8(+) T cells.

Few studies have addressed the role of these factors in the development of innate CD8(+) T cells in humans (38, 40). Interestingly, our own results (38) have shown a relation of proportionality between PLZF expression in T and iNKT lymphocytes and Eomes expression by innate CD8(+) T cells in cord blood, suggesting that iNKT cells control the differentiation of human innate CD8(+) T cells. Our results *in vitro* (41) suggest that IL-4 favors Eomes expression and the generation and/or expansion of human innate CD8(+) T cells.

IS THERE A ROLE FOR INNATE CD8(+) T LYMPHOCYTES IN CANCER IMMUNOSURVEILLANCE?

There exist only sparse data in either humans or mice describing the functions of innate CD8(+) T lymphocytes. An initial set of results consisted in a demonstration in mice of a protective role of IM/VM CD8(+) T lymphocytes against viral (30, 54) and bacterial (11) infections. Our studies have been focused on the numeric/functional status of innate CD8(+) T lymphocytes during the multistep development of human tumors.

Innate CD8(+) T Lymphocytes in Chronic Myeloid Leukemia (CML)

Similarly to iNKT cells (55–58), innate CD8(+) T cells fulfill functions providing them with anticancer potential. Hence, a deficiency of these cells in CML (**Box 1**) on diagnosis is likely to constitute, as with iNKT cells (**Box 2**), an immune subversion signature. If this is indeed the case, parallel study of iNKT cells and innate CD8(+) T cells in CML both at diagnosis and following molecular remission by tyrosine kinase inhibitor (TKI) therapy could perhaps answer questions concerning a dynamic process of generation of innate CD8(+) T cells in humans that would depend on iNKT cells.

BOX 1 | Chronic myeloid leukemia (CML), a myeloproliferative syndrome controlled by the immune system.

Chronic myeloid leukemia is the first malignant disorder with a specific genetic abnormality in the background. It is due to the formation of the chimeric oncogene BCR–ABL. This oncogene is responsible for the transformation of hematopoietic stem cells (HSC) into leukemic stem cells, which results in a leukemic syndrome of mature myeloid cells characterizing the chronic phase (CP) and ineluctably evolving, without treatment, to acute leukemia (59). The ABL domain of the chimeric oncogene BCR–ABL presents deregulated tyrosine kinase activity, which is responsible for the transformation of the HSC. Since the outset of the 2000s, new CML treatment has consisted in the tyrosine kinase inhibitors of BCR–ABL. Some arguments suggest that CML is a disease in which the immune system has a key role [for review, see Ref. (60)]. In addition, during the chronic phase of CML, numerous innate anomalies in the innate immune system have been evidenced. Indeed, defective differentiation of the plasmacytoid dendritic cells, defective IFN-α production by mononuclear cells, and defective functions of NK cells have been observed (61, 62).

BOX 2 | Functional deficiency of iNKT cells in chronic phase of CML (CML-CP) patients.

Our team has shown in CML-CP patients at diagnosis a major defect in the iNKT lymphocyte functions, particularly as concerns their proliferative response to T-cell receptor (TCR) stimulation (63) and their cytotoxic arsenal, with a loss in the expression of perforin and FasL, two elements implicated in cancer immunosurveillance by iNKT cells in mice (57, 64). It must also be emphasized that the iNKT cells of CML-CP patients have lost their expression of the transcription factor promyelocytic leukemia zinc-finger factor and no longer produce IL-4 during TCR engagement; on the other hand, they show normal expression of IFN-γ in comparison with the iNKT lymphocytes of healthy donors or patients in complete remission following treatment by tyrosine kinase inhibitors such as imatinib mesylate (63).

There exists a major defect in the innate CD8(+) T cells of chronic phase of CML (CML-CP) patients compared to those of healthy subjects or patients in complete remission following TKI treatment (41). This numerical defect is associated with a loss of IFN-γ expression after innate-like stimulation by IL-12 + IL-18 cytokines and with a loss of degranulation after stimulation *via* CD16. On the contrary, IFN-γ expression after TCR stimulation (instead of IL-12 + IL-18 stimulation) by the same innate CD8(+) T cells during CP is conserved, thereby showing that the functional defect affecting our population of interest is innate rather than adaptive. Finally, there exists a partial reconstitution in CML remission patients of the frequency and functions of innate CD8(+) T lymphocytes in terms of IFN-γ expression in response to IL-12 + IL-18 and as regards the displaying of cytotoxic functions after CD16 stimulation.

In CML patients, the numerical and functional status of innate CD8(+) T cells seems closely linked to that of the iNKT cells. In analysis of a cohort of CP patients and those in complete remission, we have observed a correlation between Eomes expression by innate CD8(+) T cells and PLZF expression by iNKT cells (41). This finding underscores the possible pathophysiological significance of IL-4 expression by iNKT cells in CML patients; while deficient during the CP, IL-4 expression is restored in remission (63) and could determine the status of innate CD8(+) T cells during the disease or its treatment.

More generally, a scenario can now be outlined as a possible explanation, during CML, for immune subversion of innate CD8(+) T cells by leukemic cells. Immune subversion could result from dysfunction of the antigen-presenting cells (APCs), particularly leukemia myeloid dendritic cells (DCs) and their environment; they might be considered as responsible for the loss of function of the innate immune cells, including NK and innate CD8(+) T cells. As regards iNKT cells, their loss of function could be due to a loss of CD1d expression by the leukemic APCs expressing the BCR–ABL oncogene [**Figure 5**; (65)]. The loss of CD1d expression could reprogram iNKT cells by favoring the loss of PLZF expression and, consequently, of IL-4, thereby decreasing their capacity to orient a differentiation of CD8(+) T cells into innate CD8(+) T cells (**Figure 5**).

Another, non-exclusive hypothesis is that of a loss of IL-15 expression by leukemic cells or of the leukemic milieu, as has been shown in colon cancer (66). A different study leads to the suggestion that during CML, TKI might favor the capacity of the DCs to trans-present IL-15 to T cells/NK cells (67). The scope of these works should be broadened, leading to a search for a defect in the expression and trans-presentation of IL-15 during CML-CP. Another relevant element is the loss in sensitivity to IL-15 or signalization of the latter by the innate CD8(+) T cells. Our preliminary results suggest a loss in response to IL-15 by total and innate CD8(+) T lymphocytes (Figure S4 in Supplementary Material). The possible implication of this loss of sensitivity to IL-15 in the dysregulation of the innate CD8(+) T lymphocytes during CML-CP remains to be investigated.

To conclude, CML can be considered as a model in study of the loss of innate effectors (NK cells) and innate T effectors [iNKT cells and innate CD8(+) T cells] and their relevance in the leukemic process. The escape of leukemic cells from the immune

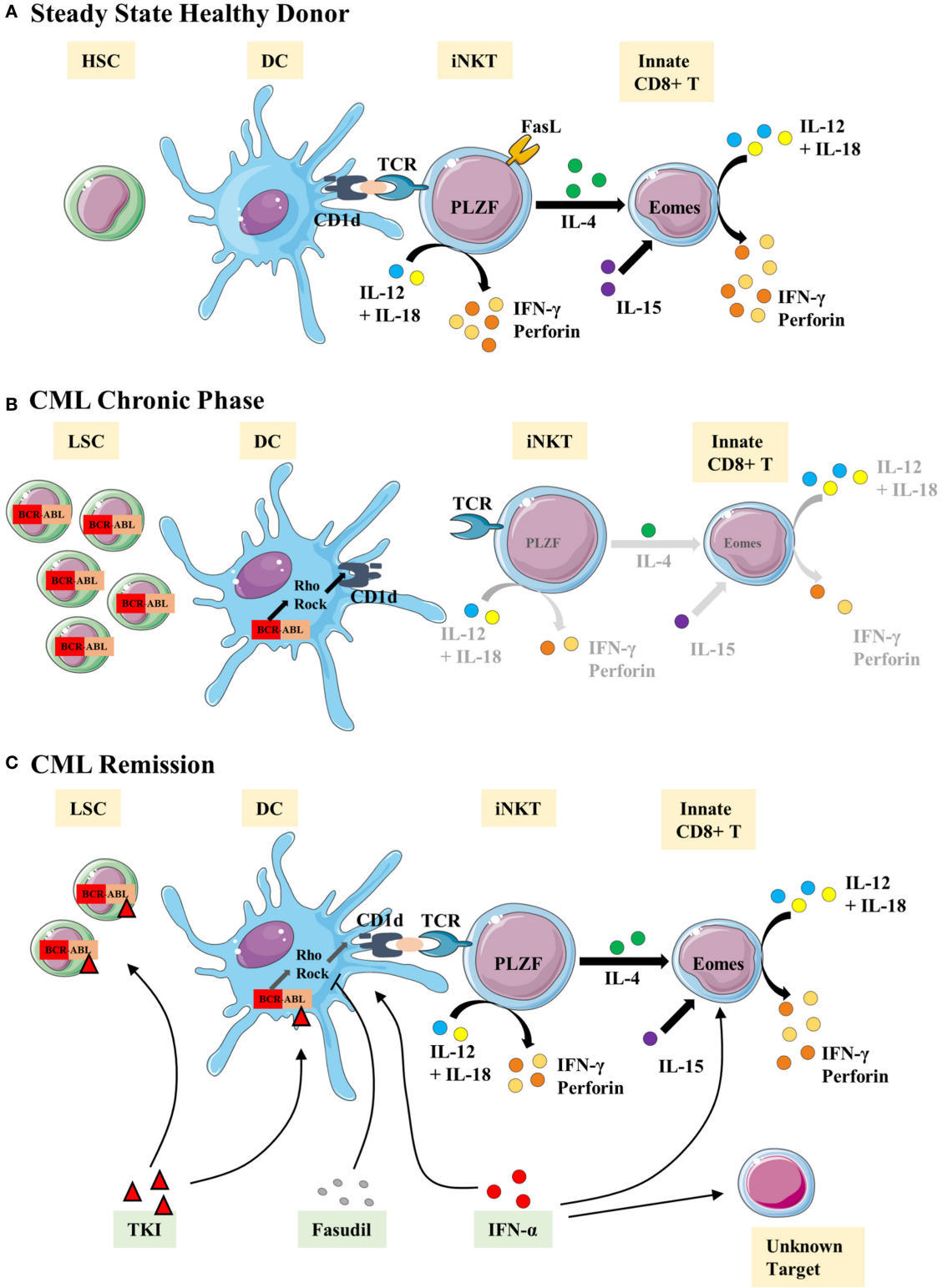
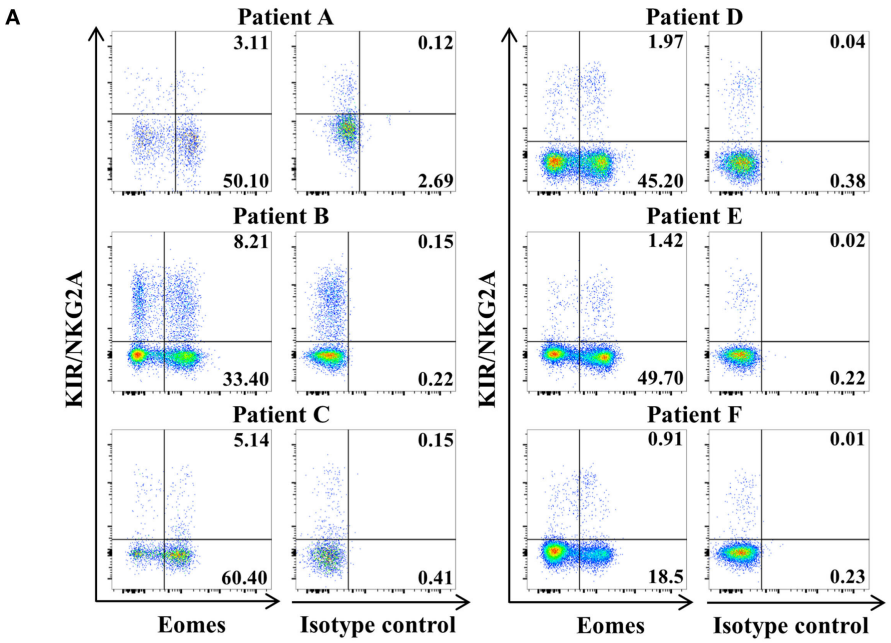


FIGURE 5 | **Continued**

FIGURE 5 | Continued

Representative view of the iNKT/innate CD8(+) T-cell axis hypothesis in chronic myeloid leukemia (CML). We propose the following scenario in CML: **(A)** steady state/healthy situation. Normal hematopoietic stem cells (HSC) generate normal immune cells. Antigens are presented *via* the CD1d molecule by dendritic cells (DCs) to iNKT cells. We propose that activated iNKT cells produce IL-4 but the possibility of a T-cell receptor (TCR)-independent mechanism for IL-4 secretion cannot be ruled out. IL-4 is thought to take part with IL-15 in the development/homeostasis of innate CD8(+) T cells. iNKT and innate CD8(+) T cells produce IFN-γ and perforin in response to the innate-like IL-12 + IL-18 stimulation. **(B)** Chronic phase of CML. Leukemic stem cells (LSC) produce modified immune cells bearing BCR–ABL translocation, including DCs. Impaired CD1d antigen presentation by DCs results from activation of the Rho/Rock pathway *via* the DH-PH domain of the ABL part of BCR–ABL. iNKT cell development/stimulation is thereby impaired, especially in terms of promyelocytic leukemia zinc-finger factor (PLZF) expression and IL-4 production. Consequently, we surmise that the innate CD8(+) T subset is defective in number and function. **(C)** Restoration of the iNKT/innate CD8(+) T-cell axis by therapies. IFN-α therapy is thought to help restoring DCs and innate CD8(+) T cells as well as other unidentified cells. Tyrosine kinase inhibitor (TKI) therapies targeting the ABL tyrosine kinase domain clear/control the generation of LSC and abnormal immune cells, including DCs. Fasudil therapy, combined with TKI, restores the CD1d presentation by DCs to iNKT cells and is one possible mechanism to restore the iNKT/innate CD8(+) T-cell axis.



B

Patient	Age	Treatment before surgery	Estrogen Receptor	Progesterone Receptor	Receptor tyrosine-protein kinase erbB-2 (HER-2)	CCI/CLI	Classification RO/RP/HER2
A	69	No	+	+	-	CCI	Luminal
B	43	Chemotherapy	-	-	-	CCI	Triple negative
C	41	No	+	+	-	CCI	Luminal
D	59	No	+	+	-	CCI	Luminal
E	66	No	+	+	-	CCI	Luminal
F	51	No	+	+	-	CCI	Luminal

FIGURE 6 | Innate CD8(+) T cells are present in tumor-draining lymph nodes (TDLNs) from breast cancer patients. **(A)** Frequencies of innate CD8(+) T cells among total CD8(+) T cells in axillary TDLNs were analyzed *ex vivo* by flow cytometry. The full cohort of five luminal breast cancer patients and one triple negative patient with chemotherapy is shown. Quadrant frequencies are displayed in upper right corners. **(B)** Clinical patient characteristics. CCI: invasive ductal carcinoma. CLI: invasive lobular carcinoma.

system could depend on loss of the coordinated functions of the effectors of classical innate immunity and innate T-cell immunity.

Innate CD8(+) T Lymphocytes and Solid Tumors

Since we cannot rule out the possibility that the anticancer role attributed to innate CD8(+) T lymphocytes is specific to leukemia, we have sought to extend the scope of our hypothesis on the antitumor role of the innate CD8(+) T lymphocytes by assessing the presence of these cells in solid tumors or metastasized tissues. The characteristics of tumor micro-environments differ according to cancers, and it is for that reason that we have assessed the presence of innate CD8(+) T lymphocytes in two types of solid tumors: breast cancer and ovarian cancer.

Study of lymph nodes (LN) invaded by tumor cells in breast cancer has highlighted the significantly frequent presence of innate CD8(+) T lymphocytes (**Figure 6**). The results of this preliminary study seem to justify organization of a large-scale study on breast cancer patients aimed at determining a possible link between, on the one hand, the numerical and functional status of the innate CD8(+) T cells present in the invaded LN draining the tumor and, on the other hand, disease prognosis.

Study of intra-tumoral lymphocytes in ovarian cancer has shown the significantly frequent presence of innate CD8(+) T lymphocytes, which are also present in the peritoneal carcinosis of ovarian cancers and in ascite fluids (**Figure 7**). Interestingly, there was a significant higher frequency of innate CD8(+) T lymphocytes in primitive tumors than in carcinosis (a proximity metastasis), indicating that these cells not only penetrate tumors in ovarian cancer but also might undergo immune subversion in the peritoneal environment. Moreover, like in CML, our data have shown a positive correlation between Eomes expression in innate CD8(+) T lymphocytes and PLZF expression in iNKT cells both in peripheral blood mononuclear cells (PBMCs) and tumor material (but not in carcinosis and ascite) from ovarian cancer patients (**Figure 7C**).

Taken as a whole, these different results show that innate CD8(+) T lymphocytes are present in tumors and probably integrated in the dynamics of anticancer responses. However, at this

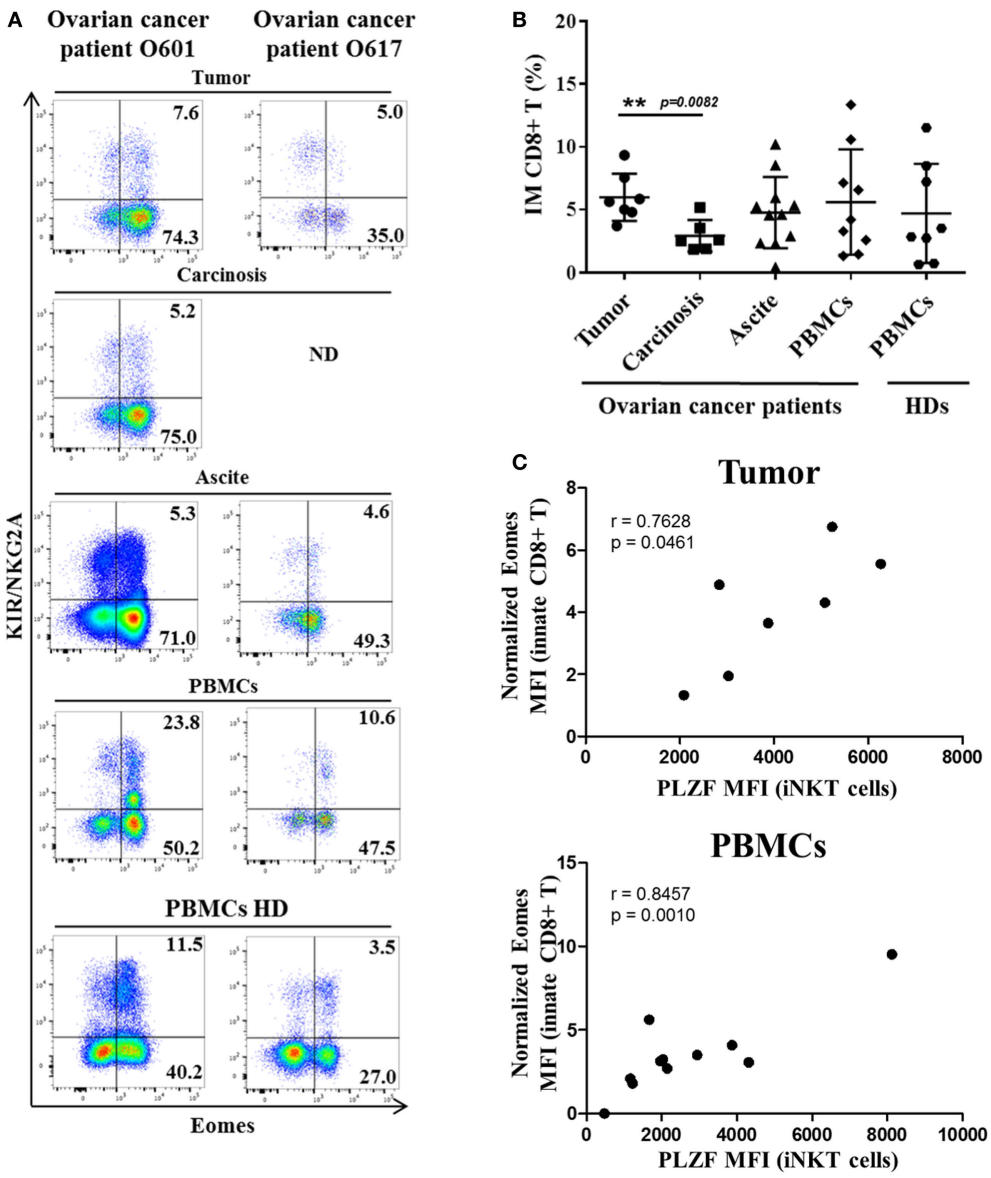


FIGURE 7 | Continued

D

Patient	Age	Histology	Grade	FIGO Stage*	Node Involvement	Ca125 (UI/L)
O601	76	Serous adenocarcinoma	High	IIIC	No lymphadenectomy	2979
O617	81	Endometrial adenocarcinoma	Low	IIIC	No lymphadenectomy	454
O596	41	Cell carcinoma arising from mature cystic teratoma	NA	Ia	No lymphadenectomy	1525
O460	52	Serous adenocarcinoma	Intermediate	IIIA	21 removed nodes / 1 node metastasis	1947
O522	72	Carcinosarcoma	NA	IIIC	62 removed nodes / No metastasis	235
O722	71	Serous adenocarcinoma	High	IIIC	83 removed nodes / 59 node metastasis	2900
O709	67	Serous adenocarcinoma	High	IV	No lymphadenectomy	1309
O622	61	Serous adenocarcinoma	High	IIIc	50 removed nodes / No metastasis	1226
O625	66	Serous adenocarcinoma	High	IIIc	35 removed nodes / No metastasis	188
O629	67	Serous adenocarcinoma	High	IIIc	37 removed nodes / 6 node metastasis	1700
O637	47	Serous adenocarcinoma	High	IIIc	30 removed nodes / 2 node metastasis	837
O670	44	Serous adenocarcinoma	High	IIIC	30 removed nodes / 1 node metastasis	837

FIGURE 7 | Innate CD8(+) T cells are present in tumors and tumor fluids from ovarian cancer patients. Cells from tumors, carcinoma, peritoneal ascites, and peripheral blood mononuclear cells (PBMCs) from ovarian cancer patients and PBMCs from healthy individuals [healthy donors (HDs)] were analyzed *ex vivo* by flow cytometry. **(A)** Two representative samples reflecting the presence of innate CD8(+) T cells in tumor, carcinoma, peritoneal ascites, and PBMCs from ovarian cancer patients and PBMCs from two HDs. ND, not determined. **(B)** Cohort study of innate CD8(+) T-cell frequency [expressed as percentage ± SD of T cells among total CD8(+) T cells] in tumors ($n = 7$), carcinoma ($n = 7$), peritoneal fluids ($n = 12$), and PBMCs from ovarian cancer patients ($n = 10$) and PBMCs from HDs ($n = 8$). **(C)** Eomes and promyelocytic leukemia zinc-finger factor (PLZF) expression were analyzed in innate CD8(+) T cells [CD3(+) CD8(+) KIR/NKG2A(+) Eomes(+) cells] and iNKT cells [CD3(+) 6B11(+) cells], respectively. Eomes MFI values are expressed relative to that of CD45(+) CD3(−) KIR/NKG2A(−) cells. The MFI of PLZF-expressing iNKT cells correlate positively with Eomes MFI in innate CD8(+) T both in tumor and PBMCs but not in carcinosis ($r = -0.04673$, $p = 0.8853$) and ascites ($r = 0.5424$, $p = 0.2084$) from ovarian cancer patients (correlation Spearman test). **(D)** Clinical patient characteristics. NA, not available.

time, it is not possible to determine their intra-tumoral functional status and, more specifically, their prognostic usefulness.

Innate CD8(+) T Lymphocytes and Immune Exhaustion in Cancer Patients

Numerous studies have dealt with the role of Eomes in the exhaustion of CD8(+) T lymphocytes during chronic infections or cancers (68–72). Our results in healthy donors (HDs) attesting a significant proliferative response to IL-15 (Figure S4 in Supplementary Material) do not lend support to an exhaustion phenotype of innate CD8(+) T cells in normal conditions. During cancer progression, on the other hand, the elevated frequency of innate CD8(+) T lymphocytes in contact with the tumor present in severely ill patients raises the question of their possible exhausted immune-exhaustion status. Furthermore, during CML, we have observed a functional defect in the innate CD8(+) T-cell population, a finding suggesting not only that CML innate CD8(+) T cells are in the process of being exhausted but also that they could be a target of immune blocking checkpoints. Monitoring multifunctionality of immune-exhausted CD8(+) T cells co-expressing Eomes and KIR/NKG2A in patients with solid tumors and CML will help to determine whether the evasion/subversion mechanisms used by tumor cells also apply to innate CD8(+) T cells in humans. At this stage, it would be interesting to determine whether the innate CD8(+) T cells express CXCR3 and CXCR5, two chemokine receptors that were recently described as being associated with CD8(+) T lymphocytes during cancers or chronic viral infection and of which the proliferation is restored following anti-programmed cell death 1 treatment (73).

CONCLUSION

Taken together, having availed ourselves of different types of empirical data, we propose that in humans, innate CD8(+) T lymphocytes constitute a new cellular component that could have a role in antitumor immunity. Our results during CML and ovarian cancer are in favor of the existence of an axis composed of innate T cells with an antitumoral potential, which consist of iNKT cells and innate CD8(+) T lymphocytes. Differentiation of these unconventional CD8(+) T cells in humans is associated with Eomes expression and could depend on IL-4 and PLZF(+) iNKT cells. Our results suggest that during CML, these innate CD8(+) T lymphocytes could be controlled by immune checkpoints. While the results of our studies on solid cancers corroborate our hypothesis concerning the role of innate CD8(+) T lymphocytes in antitumor immunity, as of now we are unable to determine whether this role is protective, permissive, and/or detrimental in these other types of cancer.

METHODS

PBMCs from HD

Healthy donors were volunteers from the Pôle Biologie Santé (Poitiers, France). PBMCs were isolated from blood samples by density gradient centrifugation (Histopaque®-1077, Sigma-Aldrich), resuspended in 90% fetal calf serum with 10% DMSO, and placed in a controlled rate freezer for cryopreservation at −80°C until use. For this series of data, age range was between 21 and 65 with a sex ratio of 0.6.

Clinical Breast and Ovarian Cancer Samples

Invaded tumor-draining lymph nodes were collected from six untreated luminal breast cancer patients undergoing standard surgery at Institute Curie Hospital (Paris, France), in accordance with institutional ethical guidelines. Precisely, all patients gave informed consent in a written form in accordance with the Declaration of Helsinki for participation in this study, which was approved by the scientific committee of the Institute Curie Hospital.

Cells from tumors, carcinomatosis, peritoneal ascites, and PBMCs were collected from eight untreated patients with ovarian carcinoma undergoing standard surgery at CHU of Rennes. Human samples were obtained from the processing of biological samples through the Centre de Resources Biologiques Santé of Rennes (BB-0033-00056). The research protocol was conducted under French legal guidelines and fulfilled the requirements of the local institutional ethics committee.

Tissue samples were cut into small fragments, digested with 0.1 mg/ml Liberase TL (Roche) in the presence of 0.1 mg/ml DNase (Roche) for 15–30 min before the addition of 20% FCS. Cells were filtered on a 40-µm cell strainer (BD), washed, and cryopreserved for further study. Ascite cells were obtained after centrifugation (400 *g*, 10 min).

Experimental Studies in Animals

The 8-to-12-week-old female C57BL/6JRj Eomes-GFP transgenic mice (74) and BALB/c wild-type mice (Janvier) were used and bred in our animal facility (PREBIOS, Platform of Research and Experimentation in Health Biology of the University of Poitiers) under specific pathogen-free conditions. Spleen and thymus were collected immediately after cervical dislocation. Splenocytes and thymocytes were isolated and analyzed *ex vivo* by flow cytometry. In some experiments, splenocytes were cultured for 16 h in the presence of 20 ng/ml of each cytokine (IL-12: R&D Systems; IL-18: MBL International) prior to analysis by flow cytometry. All procedures were performed in accordance with the recommendations of the European Accreditation of Laboratory Animal Care and French institutional committee of Poitou-Charentes (COMETHEA, C2EA-84, no. 2016072216352833).

Cell Culture and Functional Assays

Peripheral blood mononuclear cells (1×10^6 cells/ml) were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FCS and antibiotics. For IL-12 + IL-18 or IL-15 stimulation, PBMCs from HD were seeded at 1.10^6 cells/ml into 24-well plates and incubated for 48 h with 20 ng/ml of each cytokine (IL-12: R&D Systems; IL-18: MBL International; IL-15: Miltenyi). Golgistop (BD Biosciences) was added for the last 5 h of culture for IL-12 + IL-18 stimulation.

Flow Cytometry

In humans, phenotypic analysis of cells from HD or breast/ovarian cancer patients was performed by flow cytometry either *ex vivo* or after culture. Expression of different markers was assessed by staining with appropriate combinations of the following antibodies (mAbs): anti-TCR-αβ BV421 (clone: IP26, BioLegend), anti-CD8 PE-Cy7 (clone: RPA-T8, Biolegend), anti-IFN-γ FITC (clone: B27, BioLegend), anti-TCR-Vα7.2 BV421 (clone: 3C10, BioLegend), anti-CD161 PerCP-Cy5.5 (clone: HP-3G10, Biolegend), and anti-Eomes eFluor® 660 (clone: WD1928, eBiosciences). KIR/NKG2A referred to staining with the mix of the three following antibodies from Miltenyi Biotech: anti-KIR2D PE (clone: NKVFS1), anti-KIR3DL1/KIR3DL2 (CD158e/k) PE (clone: 5.133), and anti-NKG2A (CD159a) PE (clone: REA110). For nuclear Eomes staining and intracytoplasmic IFN-γ staining, cells were permeabilized with an anti-human FoxP3 staining kit (eBioscience) and a Cytofix/Cytoperm kit (BD Biosciences), respectively.

In mice, splenocytes and thymocytes were stained with appropriate combinations of the following antibodies: anti-TCR-β PerCP-Cy5.5 (clone: H57-597, BD Biosciences), anti-CD8 BV510 (clone: 53-6.7, BD Biosciences), anti-CD44 PE-Cy7 (clone: IM7, BD Biosciences), anti-CD49d Vioblue (clone: R1-2, Miltenyi Biotec), anti-CD122 APC (clone: TM-B1, Biolegend), anti-CD4 PE (clone: RM4-5, BD Biosciences), anti-CD24 PE (clone: M1/69, BD Biosciences), and anti-Eomes AF488 (clone: Dan11mag, eBioscience). For nuclear Eomes staining and intracytoplasmic IFN-γ staining, cells were permeabilized with an anti-human FoxP3 staining kit (eBioscience).

Dead cells were excluded using the Live/Dead® Fixable NearIR Dead Cell Stain kit (Life Technologies). Cells were analyzed on a Fortessa flow cytometer (BD Biosciences) or a FACSVerse™ cytometer with FACSuite™ software (BD Biosciences) using FlowJo v10 (TreeStar, Inc.).

Statistical Analysis

Statistical analyses were performed using GraphPad Prism version 7.0 (GraphPad Software). The statistical significance of differences and of mean values was analyzed by the two-tailed Wilcoxon test in **Figure 3**, paired *t*-test in **Figure 4**, and Mann–Whitney non-parametric test in **Figure 7**. Results were considered to be statistically significant when $p < 0.05$.

ETHICS STATEMENT

All patients gave informed consent in a written form in accordance with the recommendations of "the Declaration of Helsinki" for participation in the study, which was approved by the scientific committees of the Clinic Investigator Center Inserm CIC-1402 (Poitiers, France), the Biological Resource Center of CHU of Poitiers (NF S96-900 certification since February 2014), the Biological Resource Center of CHU of Rennes (NF S96-900, certification since May 2009), and the Institute Curie Hospital (Paris, France).

AUTHOR CONTRIBUTIONS

AB, EC, and FJ designed the experiments, performed the experiments, analyzed and interpreted the data, and wrote the manuscript. LL, MA, NN, NP, BM, and SB contributed to sample preparation from patients and healthy controls, designed the experiments, performed the experiments, and analyzed and

interpreted the data. EP, VC, and VL provided clinical samples and contributed to the interpretation of data. AH and J-MG together were responsible for the overall study design, supervised the project, and took primary responsibility for writing the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://journal.frontiersin.org/article/10.3389/fimmu.2017.00316/full#supplementary-material.

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