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Aging boosts antiviral CD8$^+$T cell memory through improved engagement of diversified recall response determinants

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Abstract

The determinants of protective CD8$^+$ memory T cell (CD8$^+$T$_M$) immunity remain incompletely defined and may in fact constitute an evolving agency as aging CD8$^+$T$_M$ progressively acquire enhanced rather than impaired recall capacities. Here, we show that old as compared to young antiviral CD8$^+$T$_M$ more effectively harness disparate molecular processes (cytokine signaling, trafficking, effector functions, and co-stimulation/inhibition) that in concert confer greater secondary reactivity. The relative reliance on these pathways is contingent on the nature of the secondary challenge (greater for chronic than acute viral infections) and over time, aging CD8$^+$T$_M$ re-establish a dependence on the same accessory signals required for effective priming of naïve CD8$^+$T cells in the first place. Thus, our findings reveal a temporal regulation of complementary recall response determinants that is consistent with the recently proposed "rebound model" according to which aging CD8$^+$T$_M$ properties are gradually aligned with those of naïve CD8$^+$T cells; our identification of a broadly diversified collection of immunomodulatory targets may further provide a foundation for the potential therapeutic "tuning" of CD8$^+$T$_M$ immunity.

Author summary

Immune protection provided by antiviral memory T cells relies in part on their capacity for prodigious proliferative expansion in the context of a re-infection, a proficiency that, perhaps surprisingly, is augmented rather than curtailed as memory T cells progress with age. In the present work, which draws on emerging evidence that T cell memory is not a stable trait but a progressively evolving property, we have begun to elucidate the mechanistic foundations for this age-associated amplification of memory T cell recall potential. Altogether, aged in comparison to young memory T cells more effectively engage multiple

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distinct stimulatory pathways and better eschew restraints exercised by inhibitory interactions. In this regard aged memory T cells resemble naïve T cells that depend on much of the same accessory signals to mount potent primary T cell responses. Our findings therefore provide further support for the "rebound model" of memory T cell maturation according to which aging memory T cell properties are progressively aligned with those of naïve T cells; they reveal a fundamentally temporal contingency of recall responses on the integrated activity of highly diversified molecular cues; and they identify a set of disparate targets for potential T cell-focused therapeutic interventions.

Introduction

What does it take for pathogen-specific CD8$^+$ memory T cells (CD8$^+$T$_M$) to mount an efficient and protective recall response? In most general terms, the efficacy of a secondary (II^o) CD8$^+$ effector T cell (CD8$^+$T$_E$) response is contingent on the numbers of available CD8$^+$T$_M$, their differentiation status and anatomical distribution, the contribution of other immune cell populations (*e.g.*, CD4$^+$T cells, B cells, innate immune cells), and the precise conditions of pathogen re-encounter, *i.e.* the nature of the pathogen as well as the route and dosage of infection. Thus, the specific constraints of experimental or naturally occurring pathogen exposure will dictate relevant outcomes that are predictable only in as much as the relative contribution of individual biological parameters are sufficiently understood, a task much complicated by the considerable combinatorial possibilities that ultimately shape the balance of pathogen replication and control, pathogen-induced damage, immunopathology, tissue protection and repair. Simply put, CD8$^+$T$_M$-mediated immune protection is eminently context-dependent.

The difficulties associated with attempts to define more generally applicable rules for the phenomenon of protective CD8$^+$T$_M$ immunity are perhaps best illustrated by the "effector/central memory T cell" paradigm (T$_{EM}$ and T$_{CM}$, respectively) that constitutes one of the most widely employed and consequential distinctions in the field of memory T cell research [1]. The analytical and physical separation according to CD62L (and CCR7) expression status has spawned an extraordinary amount of work that has assigned numerous distinctive, and at times seemingly contradictory, properties to CD62L^{lo} CD8$^+$T$_{EM}$ and CD62L^{hi} CD8$^+$T$_{CM}$ subsets [2–4]. The CD8$^+$T$_M$ populations thus defined, however, are very much a moving target. For example, CD62L expression by peripheral CD8$^+$T$_M$ generated in response to an acute pathogen challenge is progressively enhanced as a function of original priming conditions and infection history; upon entry into certain lymphoid or nonlymphoid tissues, CD8$^+$T$_M$-expressed CD62L is reduced; and CD8$^+$T$_{EM}$ and T$_{CM}$ subsets themselves are subject to gradual adaptations that introduce an array of molecular, phenotypic and functional changes including, importantly, an increase of their respective recall capacities [2, 5–10]. Thus, both CD62L^{lo} and CD62L^{hi} CD8$^+$T cell populations exhibit a broad spectrum of dynamically regulated properties that cannot be captured by the simple phenomenological distinction of T$_{EM}$ and T$_{CM}$ subsets. Most recently, D. Busch's group used an elegant serial adoptive transfer system in which single I^o, II^o or III^o *L. monocytogenes*- (LM-) specific CD8$^+$T$_{CM}$ (*i.e.*, CD8$^+$T$_{CM}$ established after a I^o, II^o or III^o LM challenge) gave rise to recall responses of comparable size, phenotypic and functional diversity, and protective capacity [11, 12]. Since single CD8$^+$T$_{EM}$ failed to mount a similar response, these studies provide definitive proof that the CD62L^{hi} CD8$^+$T$_{CM}$ subset harbors greater recall potential [11, 12] yet CD62L itself is apparently dispensable for an effective LM-specific recall response [13]. In some other model systems, enhanced protection was even afforded by CD8$^+$T$_{EM}$, their limited proliferative potential notwithstanding [2–4]. It

is therefore imperative to define, beyond the T_{EM}/T_{CM} paradigm, which exact mechanisms contribute to the regulation of effective CD8$^+$T$_M$ recall activity under varied experimental conditions, and to what extent specific molecular pathways may become a dominant force in a given model system. A synthesis of such efforts may then provide a foundation for the formulation of more general rules of CD8$^+$T$_M$ engagement.

In the present work, we took advantage of our observation that aging CD8$^+$T$_M$ specific for lymphocytic choriomeningitis virus (LCMV) gradually acquire unique molecular, phenotypic and functional signatures that are associated with a capacity for more vigorous II^o CD8$^+$T$_E$ responses and improved immune protection [9]. We have further organized these dynamic changes in the "rebound model" of extended CD8$^+$T$_M$ maturation according to which pertinent properties of aging CD8$^+$T$_M$ are progressively aligned, perhaps surprisingly, with those of naïve CD8$^+$T$_N$ populations [9, 10]. Here, by focusing on a diverse set of co-stimulatory and inhibitory, cytokine, chemokine and homing receptors/ligands differentially expressed by old and young CD8$^+$T$_M$ as well as their distinct effector function profiles [9], we identified a broad array of mechanisms that "tune" CD8$^+$T$_M$ recall reactivity to an acute and/or chronic viral re-challenge, and that specifically support the greater II^o CD8$^+$T$_E$ expansions of aged CD8$^+$T$_M$ populations. Collectively, our results demonstrate a novel temporal contingency of recall response determinants, and we propose in particular that aging CD8$^+$T$_M$ re-acquire a dependence on multiple accessory pathways for optimization of their II^o CD8$^+$T$_E$ reactivity that were essential for the effective and efficient priming of naïve CD8$^+$T$_N$ in the first place.

Results

Interrogating CD8$^+$T$_M$ recall responses: The mixed adoptive transfer/re-challenge (AT/RC) system

To identify the mechanisms regulating the differential recall reactivity of young and old antiviral CD8$^+$T$_M$, we employed a mixed "adoptive transfer/re-challenge" (AT/RC) system described in ref.[9]. In brief, cohorts of young adult mice congenic at the CD45 or CD90 locus were challenged with LCMV ($2x10^5$ pfu LCMV Armstrong [Arm] i.p.) and allowed to establish LCMV-specific CD8$^+$T cell memory. By performing viral infections in a staggered fashion, we generated groups of young (~2 months after challenge) and aged (>15 months after infection) LCMV-immune mice that served as donors for a concurrent interrogation of young and old CD8$^+$T cell memory. To this end, CD8$^+$T$_M$ populations were enriched from the congenic donors, combined at a ratio of 1:1 at the level of CD8$^+$T$_M$ specific for the immunodominant LCMV nucleoprotein (NP) determinant NP$_{396–404}$ (D^{b}NP$_{396}$$^+$CD8$^+T_M$), and injected into congenic recipients that were subsequently inoculated with LCMV; the respective expansions of young *vs.* old D^{b}NP$_{396}$$^+$CD8$^+T_M$-derived II^o CD8$^+T_E$ populations were then quantified eight days later (Fig 1A).

As detailed in ref.[9], the mixed AT/RC model offers several practical advantages that facilitate the elucidation of molecular mechanisms in control of differential CD8$^+$T$_M$ recall capacities. 1., young and old II^o CD8$^+$T$_E$ responses develop in the same host and are therefore subject to the same general perturbations provoked by various experimental interventions. 2., although the present analyses are for practical purposes focused on young and old D^{b}NP$_{396}$$^+$CD8$^+T_M$, their differential recall potential is a trait shared with all other LCMV-specific CD8$^+$T$_M$ populations. 3., the recall responses elaborated by transferred CD8$^+$T$_M$ populations are primarily shaped by their intrinsic properties and, importantly, are largely independent of host age. 4., the AT of low CD8$^+$T$_M$ numbers permits their maximal *in vivo* activation in the absence of artifacts that may arise from competition (*i.e.*, the mixed AT/RC approach faithfully recapitulates the extent of differential II^o expansion observed in experiments with

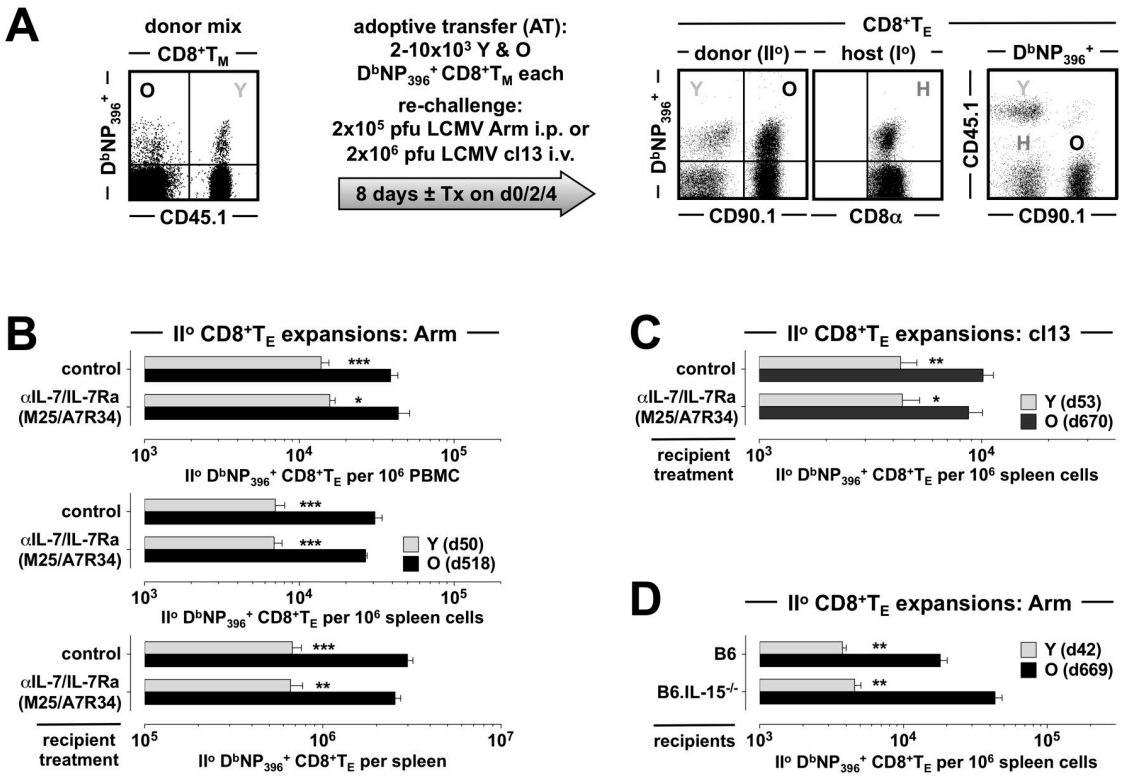

Fig 1. No role for IL-7, TSLP or IL-15 in the differential regulation of young and old IIº $CD8^+T_E$ expansions. A., basic design of mixed AT/RC experiments. $CD8^+$T cells from congenic young and old LCMV-immune donors were enriched, combined 1:1 at the level of $D^bNP_{396}{}^+CD8^+T_M$, and transferred i.v. into recipients that were subsequently challenged using "acute" (LCMV Arm) or "chronic" (LCMV cl13) infection protocols; proliferative expansions of IIº $D^bNP_{396}{}^+CD8^+T_E$ were quantified 8 days later. Note that the constellation of congenic markers permits the distinction of young and old IIº $CD8^+T_E$ as well as Iº $CD8^+T_E$ generated by the host. Unless noted otherwise, treatment with blocking antibodies was performed ~2h before AT and on d2 and d4 after virus inoculation; in other cases, B6 *vs*. immunodeficient recipients were used. **B.,** quantification of IIº $CD8^+T_E$ expansions under conditions of control (PBS) or combined αIL-7/αIL-7Ra treatment and LCMV Arm challenge; the age of donor $CD8^+T_M$ is indicated in the legend (young: d50, old: d518) **C.,** similar experiments as in panel B but conducted with LCMV cl13 and control treatment with rat IgG (donor ages indicated in legend). **D.,** mixed AT/RC experiments performed with B6 *vs*. B6.IL-$15^{-/-}$ recipients (AT of $2x10^3$ [panel B & D] or $10x10^3$ [panel C] young and old $D^bNP_{396}{}^+CD8^+T_M$ each; n≥3 mice/group; asterisks indicate significant differences comparing young and old IIº $D^bNP_{396}{}^+CD8^+T_E$ populations using Student's t-test).

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separate recipients of young and old $CD8^+T_M$). 5., similarly, the transfer of small $CD8^+T_M$ trace populations does not prevent the generation of concurrent Iº $CD8^+T_E$ responses; accordingly, the system can monitor the relatively independent evolution of three $CD8^+T_E$ populations targeting the same viral epitope (Iº, young IIº and old IIº $CD8^+T_E$ [Fig 1A]; in fact, the contemporaneous investigation of Iº $CD8^+T_E$ immunity can serve as an "internal control" since the effects of most treatment modalities employed here have a published precedent in naïve LCMV-challenged mice). 6., importantly, the relative extent of proliferative expansion of IIº $CD8^+T_E$ (but not their functional or phenotypic profiles) can serve as a correlate for immune protection. 7., the use of two different re-challenge protocols can differentiate between basic determinants required for $CD8^+T_M$ recall responses in the wake of an "acute" LCMV Arm infection (AT/RC Arm) and a more complex constellation of mechanisms supporting the effective coordination IIº $CD8^+T_E$ expansions after a "chronic" LCMV clone 13 infection (AT/RC cl13) (Fig 1A).

Altogether, we deployed the mixed AT/RC approach to ascertain the contribution of particular molecular pathways to the divergent IIº expansion of young and old $CD8^+T_M$ by

treatment of recipients with blocking antibodies or use of immunodeficient hosts (Fig 1A). While the systemic nature of these interventions cannot discern between direct and indirect effects exerted on CD8$^+$T cell populations, the broad utility and practical relevance of our approach lies in the relative ease with which CD8$^+$T$_E$ cell responses can be reliably manipulated; furthermore, in the case of antibody administration, our study design also emulates clinically relevant settings for the modulation of CD8$^+$T cell responses. Lastly, for facilitated manipulation of CD8$^+$T$_M$ we additionally employed the "p14 chimera" model in which purified congenic p14 T$_N$ (T cell receptor transgenic [TCRtg] CD8$^+$T cells specific for LCMV glycoprotein epitope GP$_{33-41}$) are transferred into B6 recipients that are subsequently challenged with LCMV Arm to generate young and old p14 T$_M$ [9] (since p14 T$_M$ are a clonotypic population, the use of donor p14 T$_M$ also effectively controls for TCR affinity/avidity as a potentially confounding variable).

No role for IL-7 and IL-15 in the differential regulation of young and old II^o CD8$^+$T$_E$ expansions

The cytokines IL-7 and IL-15 are essential for the preservation of CD8$^+$T cell memory [14] and moreover may contribute to improved recall responses by acting as "adjuvants" to boost CD8$^+$T$_E$ immunity [15, 16]. Accordingly, the increasing expression of IL-7 and IL-15 receptor components (CD127/IL-7Ra and CD122/IL-2Rb) by aging CD8$^+$T$_M$ as well as their corresponding cytokine responsiveness (refs.[9, 10] and S1 Fig) may provide a foundation for their enhanced II^o reactivity. To test this notion, we first employed our mixed AT/RC system (Fig 1A) to quantify the impact of combined IL-7/IL-7Ra blockade on the proliferative expansion of young and old II^o CD8$^+$T$_E$. As shown in Fig 1B, both overall and differential II^o CD8$^+$T$_E$ expansions after an "acute" LCMV Arm challenge were impervious to IL-7/IL-7Ra blockade; the data also illustrate that an analysis of different tissues (blood or spleen) and the use of different denominators (II^o CD8$^+$T$_E$ per 10^6 cells or total spleen cells) yields essentially similar results (Fig 1B). Likewise, IL-7/IL-7Ra blockade remained without consequences in additional mixed AT/RC experiments using the "chronic" LCMV cl13 model (Fig 1C). Our results further exclude a relevant contribution of thymic stromal lymphopoietin (TSLP) to II^o CD8$^+$T$_E$ expansions since the TSLP receptor associates with CD127 for effective signal transduction [17], and the CD127-specific antibody used in our experiments also inhibits TSLP action [18].

We further ascertained a potential role for IL-15 in our model system by conducting mixed AT/RC experiments with IL-15$^{-/-}$ recipients. Lack of IL-15, however, did not compromise the greater II^o reactivity of old CD8$^+$T$_M$ (Fig 1D); in fact, II^o expansions of aged CD8$^+$T$_M$ were somewhat increased in IL-15$^{-/-}$ as compared to B6 control mice (2.4-fold, p = 0.01; Fig 1D). Induction of a potent recall response in the absence of IL-15, despite impaired cell cycle entry of II^o CD8$^+$T$_E$ [19], is consistent with the notably robust II^o CD8$^+$T$_E$ expansions generated by LCMV-immune IL-15$^{-/-}$ mice [20] but the precise reason for the improved response of old CD8$^+$T$_M$, perhaps facilitated by greater responsiveness to other inflammatory cues, remains unclear. Nevertheless, we can conclude that neither IL-15 nor IL-7, regardless of elevated CD122 and CD127 expression by aging CD8$^+$T$_M$, contribute to their enhanced reactivity in the particular context of an LCMV-specific recall response.

Divergent requirements of IL-4, IL-6 and TGF for enhanced II^o reactivity of aged CD8$^+$T$_M$

Similar to CD127 and CD122, expression of multiple other cytokine receptors by aging CD8$^+$T$_M$ gradually increases over time with overall gains varying from the modest (CD126/IL-6Ra, CD130/IL-6ST, IL-21R, IFNAR1) to the more pronounced (CD124/IL-4Ra, TGFβRII,

CD119/IFNγR1) (S1 Fig and ref.[9]). Corresponding temporal analyses extended here to blood-borne CD8$^+$T$_M$ populations with different LCMV specificities further support the conclusion that the prolonged phenotypic CD8$^+$T$_M$ maturation is indeed a generalized and systemic phenomenon (Fig 2A/2B & S2 Fig). The kinetics of CD124, CD126 and TGFβRII expression are of particular interest since the respective signaling pathways emerged as distinctive traits in our earlier Ingenuity Pathway Analyses of aging CD8$^+$T$_M$ [9], and both IL-6 and TGFβ have been suggested to exert crucial roles in the natural history of chronic LCMV infection [21, 22]. To further assess the relation between cytokine receptor expression levels and signal transduction capacity, we briefly exposed young and old p14 T$_M$ *in vitro* to IL-4 or IL-6 and quantified phosphorylation of STAT6 and STAT3, respectively. Here, aged p14 T$_M$ indeed responded with greater STAT phosphorylation, and the re-expression of CD124 by old p14 T$_M$ at levels otherwise found only on CD8$^+$T$_N$ correlated with equal IL-4 reactivity of these populations (Fig 2B). The generally lower CD126 (and CD130 [9]) expression by CD8$^+$T$_M$, which required overall higher cytokine concentrations for effective STAT phosphorylation as compared to the IL-4 experiments, nevertheless conferred an age-dependent differential induction of pSTAT3; at the same time, IL-10-induced STAT3 phosphorylation demonstrated no differences (Fig 2B) in agreement with the stable low-level IL-10 receptor expression by aging CD8$^+$T$_M$ [9].

Despite the heightened reactivity of old CD8$^+$T$_M$ to IL-4, initial experiments performed with the mixed AT/RC Arm approach and B6 *vs*. IL-4$^{-/-}$ recipients did not reveal a role for IL-4 in the regulation of II^o CD8$^+$T$_E$ expansions (Fig 2C). In contrast, LCMV cl13 infection of IL-4$^{-/-}$ recipients resulted in an overall decrease of specific CD8$^+$T$_E$ immunity, including a 4.0-fold reduction of the splenic I^o CD8$^+$T$_E$ response ($p = 0.0056$). At the same time, the relative reduction of old II^o CD8$^+$T$_E$ expansions was more pronounced (3.4-fold, comparing B6 and IL-4$^{-/-}$ recipients), albeit only modestly so, than that of respective young II^o CD8$^+$T$_E$ populations (2.6-fold) (Fig 2D, note arrows, values [3.4x *vs*. 2.6x], and significance [asterisks]). Our findings thus add to an emerging consensus about the importance of IL-4 for the generation of effective antiviral CD8$^+$T cell immunity [23, 24] by demonstrating a requirement for IL-4 to support greater recall responses in general, and the II^o reactivity of aged CD8$^+$T$_M$ in particular; the direct correlation between CD124 expression levels of CD8$^+$T$_M$ and their recall potential as well as the similar reduction of I^o and old II^o CD8$^+$T$_E$ expansions in IL-4$^{-/-}$ mice are further consistent with predictions of the "rebound model" that a progressive alignment of CD8$^+$T$_N$ and aging CD8$^+$T$_M$ properties may translate into a reliance on similar co-stimulatory requirements [9].

IL-6 is among the most prominent cytokines induced after an LCMV infection [25] but despite the enhanced responsiveness of aged CD8$^+$T$_M$ to IL-6 stimulation (Fig 2B), the differential II^o responses of transferred young and old CD8$^+$T$_M$ were not compromised by an LCMV Arm challenge of IL-6$^{-/-}$ recipients (Fig 2C). Using the LCMV cl13 infection protocol, IL-6-deficiency imparted a very modest 1.5-fold reduction of aged but not young II^o CD8$^+$T$_E$ expansions that also mirrored a 1.4-fold decrease of the I^o response; neither finding, however, proved significant (Fig 2D) suggesting an overall more limited contribution of IL-6 to differential young and old CD8$^+$T$_M$ recall immunity. As to the potential function of TGFβ and related cytokines in the context of CD8$^+$T$_M$ aging, we earlier noted a series of marked transcriptional adaptations in the TGFβ superfamily pathway and identified a pronounced increase of TGFβRII protein expression by aging CD8$^+$T$_M$ ([9] and Fig 2B & S2 Fig). These adaptations could conceivably attenuate exuberant II^o CD8$^+$T$_E$ responses since T cell-intrinsic TGFβ signaling was proposed to constrain LCMV-specific CD8$^+$T cell immunity under conditions of persistent viral infection [21]. More recent work, however, could not demonstrate a therapeutic effect of TGFβ blockade in chronic LCMV infection [26, 27], and in

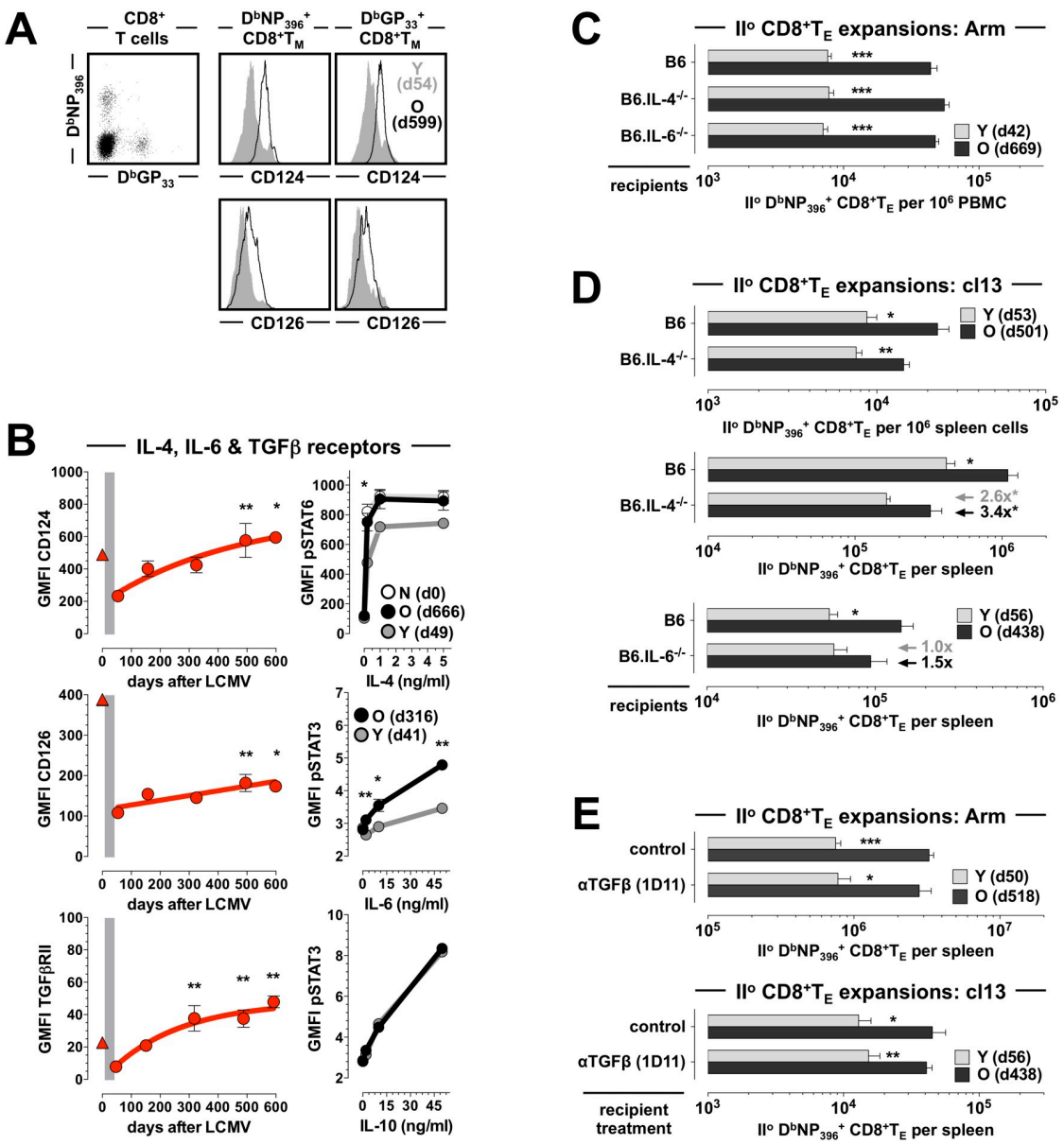

Fig 2. Divergent requirements of IL-4, IL-6 and TGFβ for enhanced II^{o} reactivity of aged CD8^{+}T$_{M}$. A., cytokine receptor expression levels by blood-borne D^{b}NP$_{396}^{+}$ and D^{b}GP$_{33}^{+}$CD8^{+}T$_{M}$ (left plot) were quantified in contemporaneous analyses of aging LCMV-immune mice by determining their respective GMFI values (geometric mean of fluorescent intensity); the overlaid histograms depict representative CD124 and CD126 expression by young (gray) and aged (black tracing) D^{b}NP$_{396}^{+}$ (middle) and D^{b}GP$_{33}^{+}$ (right) CD8^{+}T$_{M}$. **B.,** left plots: temporal regulation of CD124, CD126 and TGFβRII expression by aging D^{b}NP$_{396}^{+}$CD8^{+}T$_{M}$ (triangle symbol: CD44^{lo}CD8^{+}T$_{N}$; the gray bar demarcates the period from peak I^{o} CD8^{+}T$_{E}$ expansion [d8] to initial establishment of CD8^{+}T cell memory [d42], and asterisks indicate statistical significance comparing young and older D^{b}NP$_{396}^{+}$CD8^{+}T$_{M}$ using one-way ANOVA with Dunnett's multiple comparisons test). Right plots: STAT phosphorylation by young (gray) and old (black) p14 T$_{M}$ was assessed directly *ex vivo* and after 15min *in vitro* culture in the presence of graded dosages of recombinant IL-4 (top), IL-6 (middle) or IL-10 (bottom); the top panel also includes an analysis of p14 T$_{N}$ (white). **C.,** II^{o} CD8^{+}T$_{E}$ expansions in B6, B6.IL-4$^{-/-}$ and B6.IL-6$^{-/-}$ mice after mixed AT/RC Arm. **D.,** similar experiments as in panel C but performed with LCMV cl13. **E.,** II^{o} CD8^{+}T$_{E}$ expansions under conditions of TGFβ blockade. The gray and black arrows/values in panel D indicate the extent of significantly reduced (asterisks) II^{o} CD8^{+}T$_{E}$ expansions comparing young II^{o} CD8^{+}T$_{E}$ in B6 and B6.IL-4$^{-/-}$ mice (gray), as well as old II^{o} CD8^{+}T$_{E}$ in B6 and B6.IL4$^{-/-}$ mice (black) (n≥3 mice/group; AT of 2x10^{3} [panel C & E top], 10x10^{3} [panel D top/middle] or 5x10^{3} [panel D bottom & E bottom] young and old D^{b}NP$_{396}^{+}$CD8^{+}T$_{M}$ each).

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agreement with those studies we did not observe a further enhancement of old II^o CD8$^+$T$_E$ immunity in our mixed AT/RC system following TGFβ blockade, nor could we discern any significant impact on CD8$^+$T$_M$ recall responses *at large* in either acute or chronic infection models (Fig 2E).

Contributions of IFNγ, IFNγ receptor and FasL to the differential regulation of CD8$^+$T$_M$ recall responses

Aging of CD8$^+$T$_M$, in addition to multiple phenotypic alterations, also introduces a number of functional changes that collectively foster a more diversified spectrum of effector activities [9]. Notably, old CD8$^+$T$_M$ produce more IFNγ on a per cell basis, and a greater fraction of aged CD8$^+$T$_M$ can be induced to express Fas ligand (FasL) [9]. Together with IL-2, the production capacity of which modestly increases with age [9, 28], IFNγ and FasL also share the distinction as the only CD8$^+$T$_M$ effector molecules whose cognate receptors (CD122, CD119, CD95/Fas) are concurrently upregulated by aging CD8$^+$T$_M$ (S1 Fig and refs.[9, 10]). This can have direct implications for the autocrine regulation of CD8$^+$T$_M$ immunity in the context of recall responses as documented for IL-2 [29], and similar considerations may also apply to IFNγ given that its direct action on CD8$^+$T cells is required for optimal I^o CD8$^+$T$_E$ expansions and CD8$^+$T$_M$ development [30]. If CD8$^+$T$_M$-intrinsic FasL:Fas interactions also shape II^o CD8$^+$T$_E$ immunity, however, remains elusive.

To correlate the differential CD119 expression by young and old CD8$^+$T$_M$, confirmed and extended here to different LCMV-specific CD8$^+$T$_M$ populations in peripheral blood (Fig 3A & S2 Fig), with a direct responsiveness to IFNγ we determined the extent of STAT1 phosphorylation in young and old p14 T$_M$. Interestingly, aged p14 T$_M$ featured a slight yet significant elevation of constitutive STAT1 phosphorylation, a difference that was further amplified by *in vitro* exposure to IFNγ Fig 3A). Thus, taking into account differential CD119 expression levels, responsiveness to IFNγ, and IFNγ production capacities of young and old CD8$^+$T$_M$ [9], we conducted a first set of mixed AT/RC experiments with IFNγ$^{-/-}$. In this system, IFNγ production is restricted to the transferred CD8$^+$T$_M$ populations but both host cells and donor CD8$^+$T$_M$ can readily respond to IFNγ. Comparing CD8$^+$T$_M$ recall responses in LCMV Arm-infected B6 *vs*. IFNγ$^{-/-}$ recipients, we found that absence of host IFNγ compromised the II^o expansions of both young and old CD8$^+$T$_M$, though unexpectedly the relative decrease was more pronounced for the former rather than the latter population (Fig 3B). We therefore extended our experiments to assess the contribution of IFNγ *at large* by use of a neutralizing antibody. Here, complete IFNγ blockade further reduced II^o CD8$^+$T$_E$ responses and in particular impaired the II^o response of aged CD8$^+$T$_E$ (Fig 3B; compare the increase of relative reductions among young II^o CD8$^+$T$_E$ expansions [blood: from 2.6x in IFNγ$^{-/-}$ hosts to 2.9x after IFNγ blockade; a 1.1x increase] to those of aged II^o CD8$^+$T$_E$ populations [blood: from 1.5x in IFNγ$^{-/-}$ hosts to 2.4x after IFNγ blockade; a 1.6x increase]). Together, our findings demonstrate a moderate role for IFNγ in the regulation of CD8$^+$T$_M$ recall responses to an acute LCMV challenge that differs according to the cellular source of IFNγ: while the II^o expansion of young CD8$^+$T$_M$, despite reduced CD119 expression and signaling, is more reliant on IFNγ production by other cells, aged CD8$^+$T$_M$ populations, on account of enhanced IFNγ production capacity [9], can better promote their own II^o reactivity. This notion is further reinforced by mixed AT/RC experiments using LCMV cl13 infection under conditions of IFNγ blockade. As shown in Fig 3C, neutralization of IFNγ profoundly depressed II^o CD8$^+$T$_E$ immunity and largely extinguished any differences between young and old II^o CD8$^+$T$_E$ expansions (note the comparable population sizes in blood [top] and at the level of total II^o CD8$^+$T$_E$ per spleen [bottom] in Fig 3C).

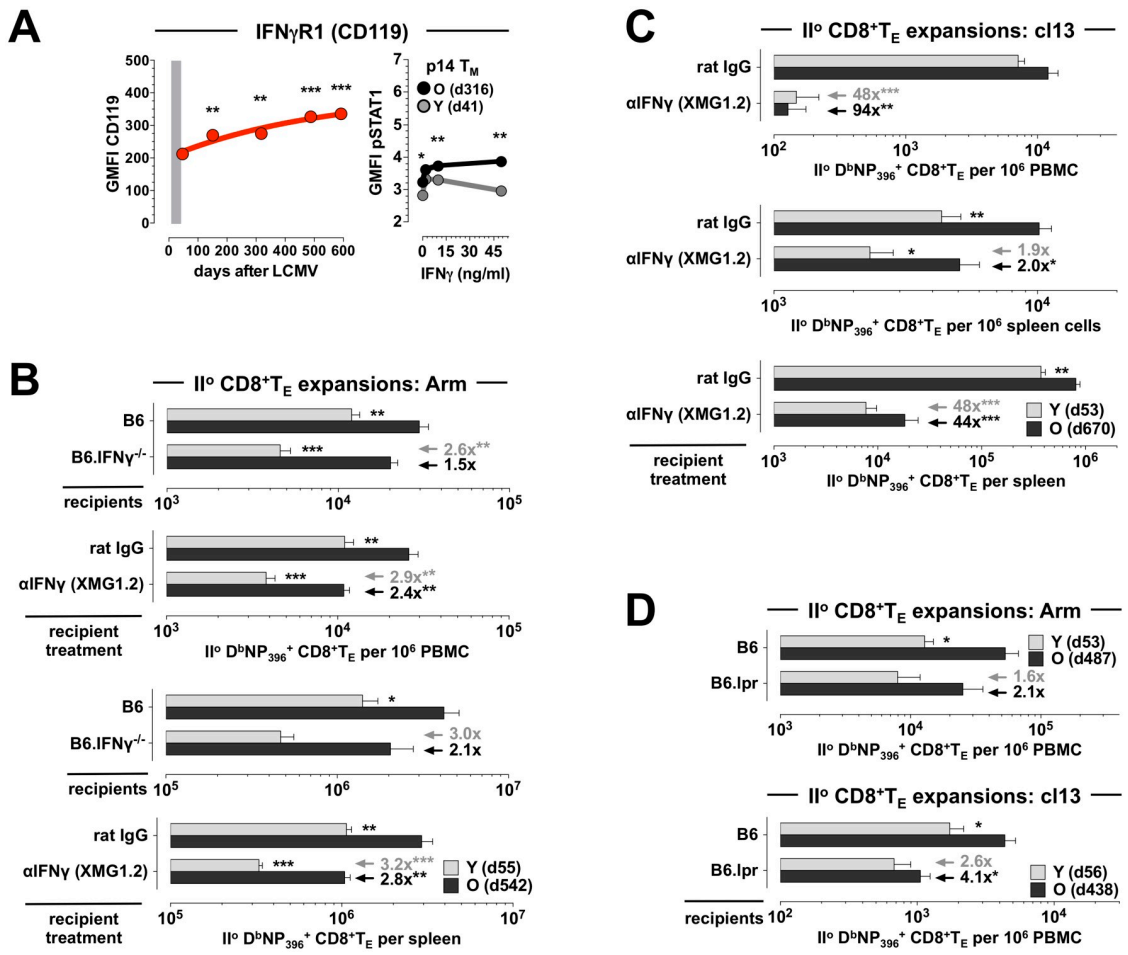

Fig 3. Role of IFNγ, IFNγ receptor and FasL in the regulation of young and old CD8$^+$T$_M$ recall activity. A., left: expression of CD119 by aging D^{b}NP$_{396}$$^+$CD8$^+T_M$ in the PBMC compartment. Right: STAT1 phosphorylation by young and aged p14 T$_M$ was determined *ex vivo* and after 15min *in vitro* exposure to recombinant IFNγ; note the slightly enhanced *ex vivo* pSTAT1 levels in old *vs.* young p14 T$_M$. **B.,** mixed AT/RC Arm experiments were performed with B6 and B6.IFNγ$^{-/-}$ recipients as well as under conditions of control (rat IgG) or αIFNγ treatment. **C.,** similar IFNγ blocking experiments as in panel B but conducted with the chronic LCMV cl13 model. **D.,** II^o CD8$^+$T$_E$ expansions after AT/RC Arm (top) or AT/RC cl13 (bottom) using B6 *vs.* B6.lpr recipients. Arrows/values in panel B-D indicate the respective extent and significance (asterisks) by which antibody treatment or immunodeficiency reduced II^o expansions of young (gray) or old (black) II^o CD8$^+$T$_E$ populations (n≥3 mice/group or time point; AT of 3x10^3 [panel B], 10x10^3 [panel C], 2x10^3 [panel D top] or 5x10^3 [panel D bottom] young and old D^{b}NP$_{396}$$^+$CD8$^+T_M$ each).

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In contrast to IFNγ, the role of FasL:Fas interactions in the LCMV model appears more limited—both FasL- and Fas-mutant mice (FasL^{gld} and Fas^{lpr} strains, respectively) control an acute LCMV infection [31]—yet a non-redundant role for Fas in virus clearance or CD8$^+$T$_M$ generation could be readily demonstrated in mice with compound immunodeficiencies [32–34]. To evaluate the contribution of the FasL:Fas pathway in our model system, we conducted mixed AT/RC experiments with B6 *vs.* Fas^{lpr} ("B6.lpr") recipients and observed a preferential reduction of aged II^o CD8$^+$T$_E$ expansions in the B6.lpr hosts that was especially pronounced following chronic LCMV cl13 infection (Fig 3D). Although we can conclude that the enhanced II^o reactivity of old CD8$^+$T$_M$ is in part controlled by their broader FasL induction, the precise mechanisms operative in this context remain to be elucidated and may involve accelerated virus clearance [9] through FasL-dependent cytolysis, nonapoptotic FasL:Fas interactions between CD8$^+$T$_M$ and T$_N$ that facilitate concurrent I^o CD8$^+$T$_E$ differentiation [35], or perhaps

the autocrine binding of secreted FasL that, akin to a mechanism proposed for tumor cells [36], may shield II^o CD8$^+$T$_E$ from FasL-mediated fratricide.

LFA-1 and CXCR3 blockade preferentially curtail II^o expansions of aged CD8$^+$T$_M$

Among the array of phenotypic changes accrued during CD8$^+$T$_M$ aging we previously noted and interrogated several cell surface receptors involved in the regulation of CD8$^+$T cell trafficking [9, 10]. Now, using an unbiased approach based on time series gene set enrichment analyses (GSEA) of aging p14 T$_M$ populations [10], the potential importance of differential "homing receptor" expression was further supported by our identification of the "cell adhesion molecules" module as the top Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway negatively enriched in old p14 T$_M$ (normalized enrichment score: -1.82; p = 0.0078; Fig 4A). For 29/38 genes within this module, we also performed temporal protein expression analyses and demonstrated a significant up- or downregulation by aging CD8$^+$T$_M$ for half of these gene products (15/29; Fig 4A and ref.[9]). Here, the expression pattern of CD11a/integrin α_L caught our attention for several reasons: elevated CD11a expression, similar to CD44, has long been used as a surrogate marker for "antigen-experienced" CD8$^+$T cells [37]. In combination with CD18/integrin β_2, CD11a forms the heterodimeric LFA-1 complex that constitutes, together with its ligands CD54/ICAM1 and CD102/ICAM2, one of the major pathways for leukocyte adhesion. In contrast to CD44, however, CD11a mRNA and protein expression by aging CD8$^+$T$_M$ are subject to a slight yet significant decline (Fig 4A/4B, S2 Fig and ref.[9]). In fact, other components of the LFA-1 pathway exhibited very similar patterns with a progressive decrease of CD8$^+$T$_M$-expressed CD18, CD102 and in particular CD54 mRNA and/or protein (Fig 4A/4B, S2 Fig and ref.[9]).

LFA-1 biology has been characterized in great detail [38, 39] but the precise role of CD11a in the regulation of pathogen-specific T cell immunity remains incompletely defined. In one of the most detailed report to date, Bose *et al.* found that CD11a-deficiency reduces I^o but paradoxically enhances II^o bacterium-specific CD8$^+$T$_E$ expansions [40]. The latter finding, however surprising, is consistent with the "rebound model" of CD8$^+$T$_M$ de-differentiation [9, 10] in that any deficits conveyed by CD11a-deficiency are eclipsed by an advanced maturation stage of CD11a$^{-/-}$ CD8$^+$T$_M$ [40] that is associated with greater recall capacity. In the LCMV system, LFA-1 blockade similarly resulted in a ~2-fold reduction of I^o CD8$^+$T$_E$ expansions ([41]) (also recapitulated in our model) but its potential impact in the specific context of CD8$^+$T$_M$ recall responses has not yet been determined. As based on the experience with LFA-1 blockade in transplantation and autoimmunity [42, 43], and considering in particular the lower CD11a and CD54 expression of CD8$^+$T$_N$ [9], we speculated that CD8$^+$T$_M$ would be overall more resistant to LFA-1 blockade but that declining CD11a and CD54 levels by aging CD8$^+$T$_M$ (Fig 4A/4B & S2 Fig) might render them again somewhat more susceptible to this intervention. Using our mixed AT/RC system, LFA-1 blockade in the context of an LCMV cl13 infection indeed promoted a prominent and preferential reduction of aged as compared to young II^o CD8$^+$T$_E$ responses in peripheral blood (4.0-fold *vs.* 2.7-fold) that was less evident in the spleen or after LCMV Arm challenge (Fig 4C and not shown). In fact, blocking LFA-1 in the chronic infection model compromised old CD8$^+$T$_M$ recall responses to an extent that approached the decrease observed for concurrent I^o CD8$^+$T$_E$ responses (4.1-fold [p = 0.0003] and 2.0-fold [p = 0.04] reduction in blood and spleen, respectively). The efficacy of LFA-1 blockade therefore correlates inversely with expression levels of CD11a (and other components of the LFA-1 pathway) on CD8$^+$T cells such that the inhibition of proliferative expansion is greater for CD8$^+$T$_N$ than CD8$^+$T$_M$, and more substantial for old than young CD8$^+$T$_M$. We conclude that

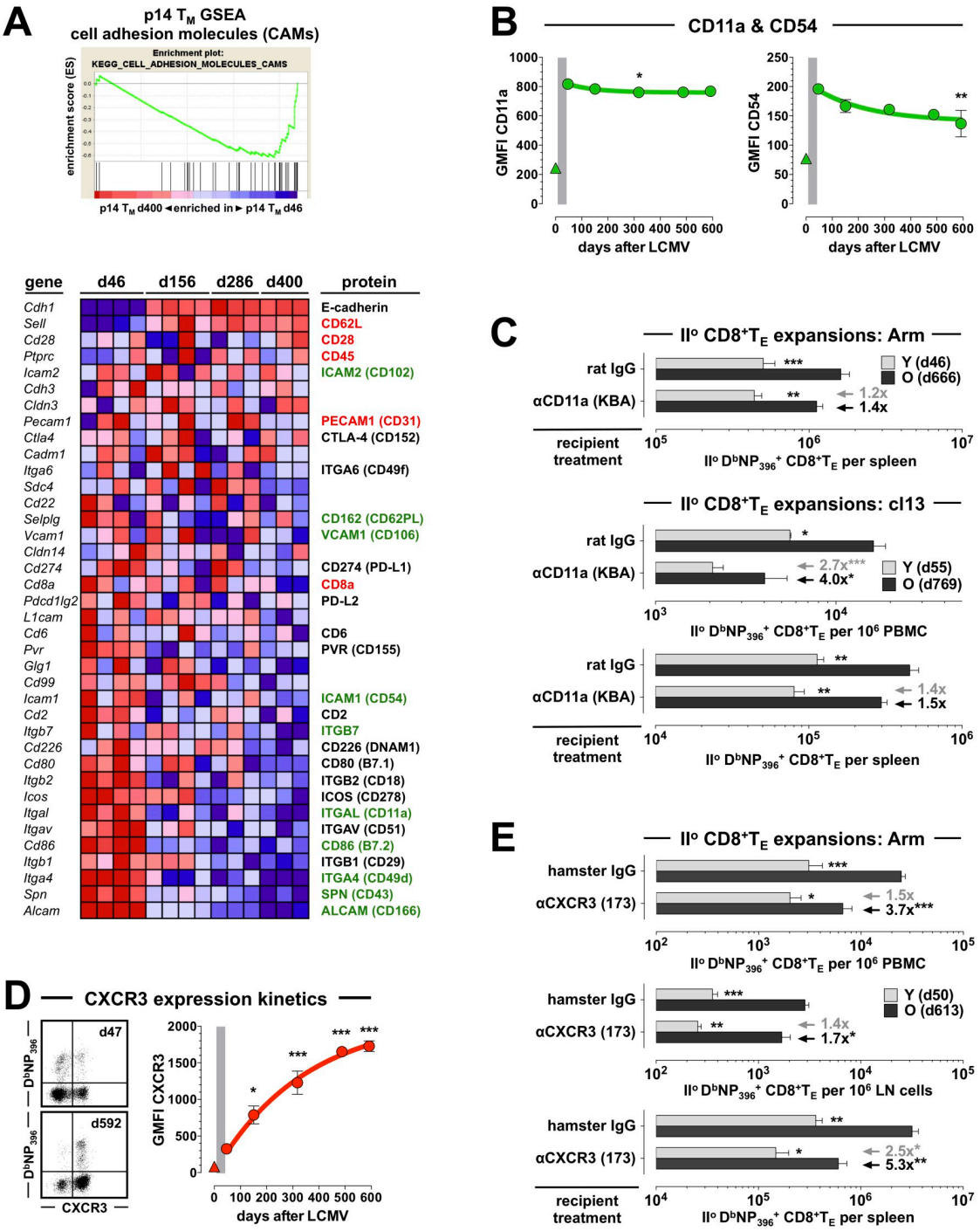

Fig 4. CD11a and CXCR3 blockade preferentially restrict IIº expansions of aged antiviral CD8+T$_M$. A., time series GSEA were conducted for *ex vivo* purified aging p14 T$_M$ (d46, d156, d286 and d400) as detailed in refs.[9, 10]. Top: old p14 T$_M$ are depleted for genes within the KEGG CAMs pathway module (NES: -1.82; p = 0.0078). Bottom: heat map displaying relative expression of individual genes by aging p14 T$_M$ (blue: low, red: high). The right hand column summarizes corresponding protein expression by aging splenic or blood-borne D^{b}NP$_{396}^+$ and/or D^{b}GP$_{33}^+$CD8$^+$T$_M$ retrieved from LCMV-immune B6 mice; colors indicate significant expression changes accrued over time (red: upregulation; black: no change; green: downregulation) and the primary protein expression data in this summary is detailed in panel B as well as Fig 5A, S2 Fig and/or refs.[9, 10]. **B.,** temporal regulation of CD11a and CD54 expression by D^{b}NP$_{396}^+$ CD8$^+$T$_{E/M}$ (triangle symbol: CD44^{lo} CD8$^+$T$_N$). **C.,** mixed AT/RC Arm and cl13 experiments were performed with rat IgG (control) or αCD11a blocking antibodies; due to the efficacy and prolonged half-life of the KBA antibody, antibodies were only injected on d0 and d2. **D.,** temporal regulation of CXCR3 expression by aging blood-borne

$D^bNP_{396}{}^+CD8^+T_M$ (dot plots gated on $CD8^+$T cells). **E.,** II^o $CD8^+T_E$ expansions in different tissues as assessed after LCMV Arm infection and control (hamster Ig) *vs.* αCXCR3 treatment. Note the preferential decrease of aged II^o $CD8^+T_E$ expansions in the wake of CD11a or CXCR3 blockade as indicated by black and gray arrows/values (panel C & E) (n≥3 mice/group or time point; AT of $5x10^3$ [panel C top & E] or $8x10^3$ [panel C middle/bottom] young and old $D^bNP_{396}{}^+CD8^+T_M$ each).

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aged $CD8^+T_M$ populations rely in part on the LFA-1 system to support their improved recall responses in the periphery.

Like the integrins, and often in conjunction, chemokine receptors sensitize T cells to essential spatiotemporal cues that effectively orchestrate developing T cell responses [44]. For example, a consensus about the importance of T cell-expressed CXCR3 for effective $CD8^+T_E$ priming and memory development has been established by multiple independent studies [45–49] yet in regard to its relevance for the regulation of II^o responses, strikingly different conclusions were reached: CXCR3-deficiency either improved [46], did not affect [47], or compromised II^o $CD8^+T_E$ reactivity [49]. The use of different model systems and experimental protocols may have contributed to the divergent outcomes but another factor may be the precise timing of re-challenge experiments since CXCR3 expression by splenic and blood-borne virus-specific $CD8^+T_M$ changes substantially over a period of ~18 months ([9, 50] and Fig 4D & S2 Fig). To provide a first orientation about the principal recall potential of $CXCR3^{hi}$ *vs.* $CXCR3^{lo}$ $CD8^+T_M$ subsets, we sorted these populations from young LCMV-immune donors and monitored the respective development of II^o $CD8^+T_{E/M}$ populations in the AT/RC Arm model. Throughout the course of the II^o response, the progeny of $CXCR3^{hi}$ $CD8^+T_M$ proved superior at the level of II^o $CD8^+T_{E/M}$ abundance and accelerated phenotypic maturation (S3A–S3D Fig) but an important caveat pertains to the fact that the $CXCR3^{hi/o}$ $CD8^+T_M$ donor subsets presented with numerous phenotypically distinctive properties beyond their CXCR3 expression status (S3D Fig). Therefore, to circumvent confounding factors associated with differential subset composition or the generation of $CXCR3^{-/-}$ $CD8^+T_M$ [47], we used a non-depleting CXCR3 antibody [51, 52] in the context of our AT/RC studies, and our results demonstrate that CXCR3 is indeed required for optimal II^o $CD8^+T_E$ reactivity. Specifically, CXCR3 blockade preferentially weakened the II^o response of old as compared to young $CD8^+T_M$, did so in a systemic fashion (i.e. was observed in blood, spleen and lymph nodes [LNs]), and to an extent that somewhat exceed the impairment of contemporaneous I^o $CD8^+T_E$ expansions (2.2-fold [p = 0.0005] and 3.5-fold [p = 0.0035] decrease in blood and spleen, respectively) (Fig 4E). Ready access for $CD8^+T_M$ to local regions of CXCR3 ligand (CXCL9/10) expression [46–49] therefore constitutes an important parameter for the optimal systemic expansion of II^o $CD8^+T_E$ populations, and aged $CD8^+T_M$, by virtue of enhanced CXCR3 expression, are poised to more effectively harness these interactions.

CD28- but not CD27-dependent co-stimulation preferentially promotes enhanced II^o reactivity of aged $CD8^+T_M$

Recall responses are traditionally regarded as "co-stimulation independent" but more recent work has documented an important role especially for CD28 in the regulation of pathogen-specific II^o $CD8^+T_E$ immunity [53]. Although our original analysis of genes differentially expressed by young and old $CD8^+T_M$ included few members of the major co-stimulatory B7 and TNF superfamilies [9], the temporal GSEAs conducted here captured many more subtle alterations, including an upregulation of *Cd28* by aging p14 T_M (Fig 4A). A corresponding age-associated augmentation of CD28 protein expression was confirmed and extended here to blood-borne $D^bNP_{396}{}^+$ and $D^bGP_{33}{}^+CD8^+T_M$ populations, and similar experiments corroborated a particularly prominent increase for CD27 (Fig 5A & S2 Fig), a co-stimulatory receptor

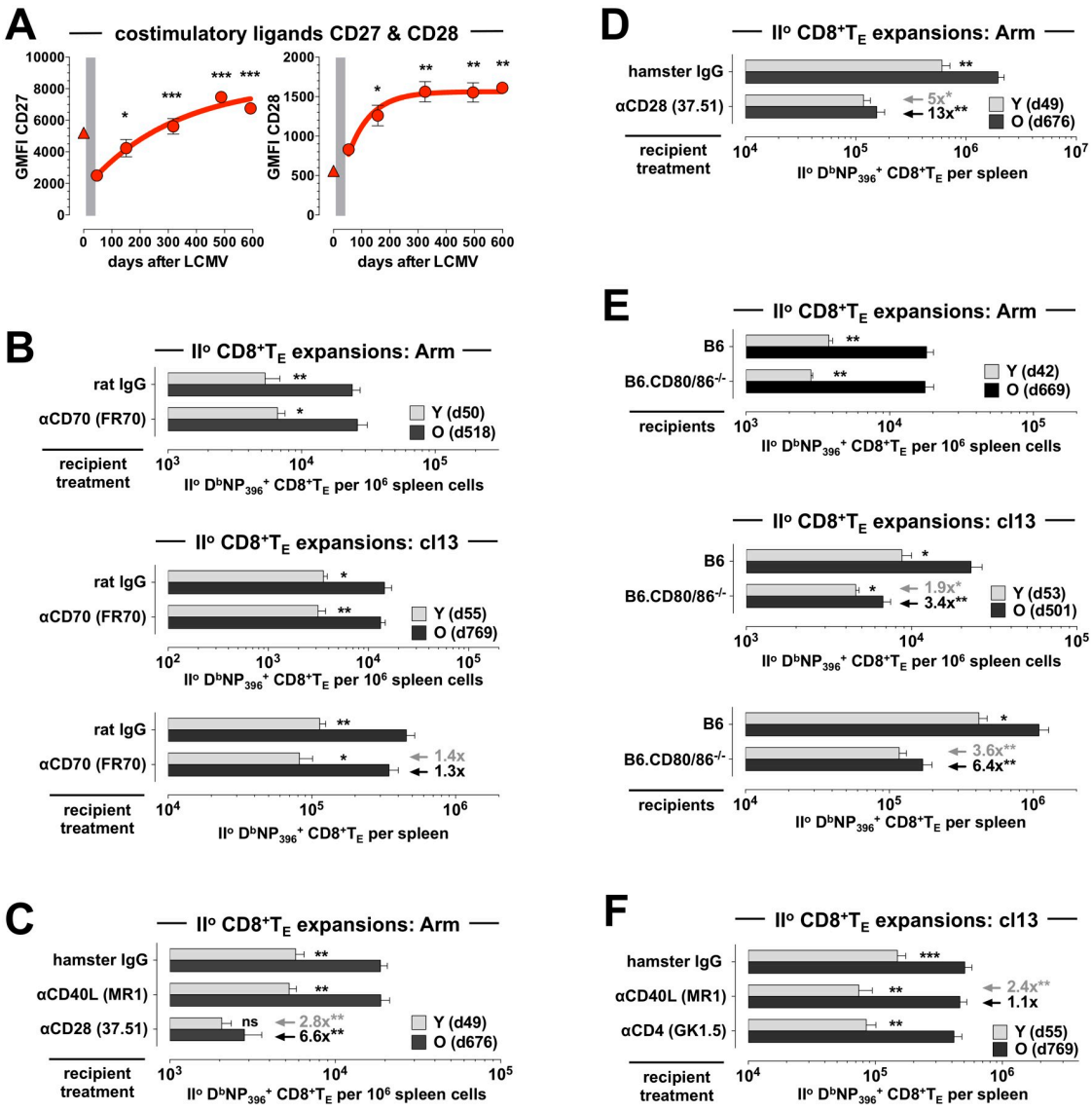

Fig 5. CD28:CD80/86 but not CD27:CD70 or CD40L:CD40 co-stimulatory interactions preferentially promote improved IIº reactivity of aged antiviral CD8⁺T$_M$. A., temporal regulation of CD27 and CD28 expression by aging D^{b}NP$_{396}$$^+$CD8$^+T_M$ in peripheral blood (triangle symbol: CD44^{lo}CD8$^+$T$_N$). **B.,** mixed AT/RC experiments were conducted in the LCMV Arm and cl13 systems as indicated using treatment with rat IgG (control) or CD70 blocking antibodies. **C.,** IIº CD8$^+$T$_E$ expansions after mixed AT/RC Arm performed under conditions of CD40L or CD28 blockade. **D.,** same experiment as in C but depicting total splenic IIº CD8$^+$T$_E$ numbers accumulated in the absence *vs.* presence of CD28 blockade. **E.,** mixed AT/RC experiments with CD80/86$^{-/-}$ recipients employing LCMV Arm (top) or cl13 (middle/bottom) infection protocols. **F.,** mixed AT/RC cl13 experiments were conducted under conditions of hamster IgG treatment (control), CD40L blockade or CD4$^+$T cell depletion. The gray and black arrows/values in panels B-D emphasize the extent of reduced IIº CD8$^+$T$_E$ expansions comparing young IIº CD8$^+$T$_E$ populations of control and antibody treated mice (gray), and old IIº CD8$^+$T$_E$ populations of control and antibody treated mice (black); adjacent asterisks indicate statistical significance (n≥3 mice/group or time point; AT of 2x10^3 [panel B top, C, D & E top], 8x10^3 [panel B middle/bottom & F] or 10x10^3 [panel E middle/bottom] young and old D^{b}NP$_{396}$$^+$CD8$^+T_M$ each).

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that exhibits some of the most pronounced expression differences between young and old CD8$^+$T$_M$ [9].

Despite the general importance of the CD27:CD70 co-stimulatory pathway [54], its contribution to the regulation of LCMV-specific CD8$^+$T$_E$ immunity appears to be more limited. CD70 blockade or deficiency modestly reduced LCMV-specific I^o CD8$^+$T$_E$ expansions after an

acute virus challenge but left the II^o response largely intact [55–57]. We made near identical observations in our mixed AT/RC Arm model conducted under conditions of CD70-blockade, *i.e*. we found a small reduction of I^o host CD8$^+$T$_E$ responses whereas the overall and differential expansions of young and old II^o CD8$^+$T$_E$ populations were fully preserved (Fig 5B). Blocking CD70 in the context of a chronic or high-dose LCMV infection, however, was reported to promote the opposite effect of modestly increasing I^o but decreasing II^o CD8$^+$T$_E$ responses [56, 58]. Again, these results were essentially reproduced in our experiments where LCMV cl13-induced I^o CD8$^+$T$_E$ host responses under conditions of CD70 blockade were somewhat elevated (~1.6-fold) yet concomitant young and old II^o CD8$^+$T$_E$ expansions were both slightly reduced (Fig 5B). Regardless of the relatively small impact exerted by CD70-blockade on the coordination of CD8$^+$T$_E$ cell immunity, the divergent regulation of I^o and II^o CD8$^+$T$_E$ responses in the same microenvironment indicates that CD27:CD70-mediated interactions are not only contingent on pathogen virulence, tropism, persistence and related parameters [54, 55] but also on the differentiation stage of specific CD8$^+$T cells themselves. At the same time, the large increase of CD27 expression by aging CD8$^+$T$_M$ remained unexpectedly inconsequential for the regulation of their II^o reactivity.

With regard to the gradual increase of CD28 expression by aging CD8$^+$T$_M$ (Figs 4A, 5A & S2 Fig), earlier work by us and others has already implicated the CD28:CD80/86 pathway in the regulation of LCMV-specific II^o CD8$^+$T$_E$ immunity [59, 60] raising the possibility that a more efficient use of these interactions by old CD8$^+$T$_M$ may boost their recall responses. In confirmation of this prediction, the impairment of II^o CD8$^+$T$_E$ expansions after CD28-blockade in the mixed AT/RC Arm scenario was more pronounced for old as compared to young CD8$^+$T$_M$ (13-fold *vs*. 5-fold) and resulted in the obliteration of any numerical differences between young and old II^o CD8$^+$T$_E$ populations (Fig 5C and 5D). An accompanying ~3.5-fold decrease of I^o NP$_{396}$-specific host populations essentially replicated the phenotype of LCMV-challenged CD28$^{-/-}$ mice [61] and the apparently lesser impact of CD28-blockade on I^o CD8$^+$T$_E$ responses may be due to the lower CD28 expression by CD44^{lo}CD8$^+$T$_N$ (Fig 5A). Using an alternative approach to probe the CD28:CD80/86 pathway, we conducted mixed AT/RC experiments with CD80/86$^{-/-}$ recipients. Based on our previous work, we anticipated a critical difference employing LCMV Arm *vs*. cl13 re-challenge protocols: despite the reliance of CD8$^+$T$_M$ recall responses on CD28, re-challenge with LCMV Arm proved independent of CD80/86 suggesting the existence of another CD28 ligand; in contrast, II^o CD8$^+$T$_E$ expansions were clearly CD80/86-dependent following an LCMV cl13 re-challenge [60]. In agreement with these findings, neither II^o nor concurrent I^o CD8$^+$T$_E$ responses elicited in the mixed AT/RC Arm system were affected by CD80/86-deficiency (Fig 5E). Yet an LCMV cl13 infection not only reduced overall CD8$^+$T$_M$ recall reactivity but preferentially comprised the accumulation of aged (6.4-fold) as compared to young (3.6-fold) II^o CD8$^+$T$_E$ (Fig 5E). Together, these results support the notion that CD28-mediated co-stimulation contributes to the regulation of CD8$^+$T$_M$ recall responses in general and to the improved II^o reactivity of aged CD8$^+$T$_M$ in particular.

Role of CD40L and CD4$^+$T cells in the differential regulation of young and old II^o CD8$^+$T$_E$ responses

In extension of our investigation into major co-stimulatory pathways above, we also evaluated the potential involvement of CD40L:CD40 interactions in the regulation of II^o CD8$^+$T$_E$ immunity, experiments prompted by our observation that aged CD8$^+$T$_M$ synthesize larger amounts of CD40L upon re-stimulation [9]. Although CD8$^+$T cell-produced CD40L appears dispensable for I^o CD8$^+$T$_E$ responses [62], it readily promotes DC activation, B cell proliferation and

antibody production [63], and may boost II^o CD8$^+$T$_E$ immunity under conditions of limited inflammation [64]. Similarly, our previous work has documented that CD40L blockade administered within the first week of acute LCMV Arm infection does not impinge on I^o CD8$^+$T$_E$ responses but affects subsequent CD8$^+$T$_M$ development as revealed by impaired II^o *in vitro* cytotoxic T lymphocyte (CTL) activity [65]. While these results point towards a more limited and context-dependent role for CD8$^+$T cell-produced CD40L, any interpretation of outcomes observed after anti-CD40L treatment has to consider that it targets both CD4$^+$ and CD8$^+$T cell subsets.

In the mixed AT/RC Arm setting employed here, acute CD40L blockade did not compromise I^o or II^o CD8$^+$T$_E$ responses (Fig 5C), observations that are also consistent with the finding that neither I^o nor II^o p14 T$_E$ responses benefitted from the provision of additional CD4$^+$T cell help [66]. Yet the situation was reportedly different in the chronic LCMV model: supplementary CD4$^+$T cell help increased II^o but not I^o p14 T$_E$ responses, and the effect was abolished by CD40L blockade indicating that CD8$^+$T$_M$ are more reliant than CD8$^+$T$_N$ on CD40L-mediated CD4$^+$T cell help [66]. In the experiments shown in Fig 5F, we further quantified CD8$^+$T$_E$ expansions after mixed AT/RC cl13 under conditions of CD40L blockade. Similar to West *et al*. [66], we found no obvious impact on I^o CD8$^+$T$_E$ responses but readily observed a significant reduction of young II^o CD8$^+$T$_E$ populations; nearly identical results were obtained when the experiments were performed with CD4$^+$T cell-depleted recipients (Fig 5F). Although these results fail to identify a specific contribution for CD8$^+$T$_M$-expressed CD40L to the regulation of recall responses, they confirm the notion of CD40L:CD40 interactions as an accessory pathway for the optimal elaboration of II^o but not I^o CD8$^+$T$_E$ responses. Perhaps most interesting is the fact that aged II^o CD8$^+$T$_E$ reactivity, just like I^o CD8$^+$T$_E$ responses, remained largely unperturbed by either CD40L-blockade or absence of CD4$^+$T cell help in the AT/RC cl13 model (Fig 5F). This outcome is in fact predicted by the "rebound model" of CD8$^+$T$_M$ maturation which proposes a progressive harmonization of aging CD8$^+$T$_M$ properties with those of CD8$^+$T$_N$ [9, 10], and thus over time a waning importance for CD4$^+$T cell help. The model can also explain the seemingly contrasting conclusion that LM-specific CD8$^+$T$_M$ recall responses become *more* CD4$^+$T cell-dependent with age [67]: as opposed to the CD4$^+$T cell-independent LCMV Arm system, Marzo *et al*. employed an LM infection protocol where CD4$^+$T cell depletion greatly reduced I^o CD8$^+$T$_E$ responses [67]. The complementary observation that CD4$^+$T cell depletion in the context of an LM re-challenge also curtailed II^o CD8$^+$T$_E$ expansions, and that this effect became more pronounced with advancing age [67] further indicates that aging CD8$^+$T$_M$ gradually re-establish a reliance on CD4$^+$T cell help akin to that exhibited by CD8$^+$T$_N$.

Enforced SAP expression constrains II^o CD8$^+$T$_E$ expansions

The expression patterns of CD2/SLAM family genes and proteins provide yet another example for the converging temporal regulation of CD8$^+$T$_M$ properties within a defined molecular family: mRNA and/or protein expression of CD2 and signaling lymphocytic activation molecule family (SLAMF) members 1/2/4-7 are all progressively downmodulated in aging CD8$^+$T$_M$ [9]. The functional relevance of these phenotypic changes, however, is difficult to predict due to the complexities, redundancies and context-dependent activities of integrated SLAMF receptor signaling [68]. Even so, since all T cell-expressed SLAMF receptors operate through the same small adaptor SAP (SLAM-associated protein) [68], a slight decline of *Sh2d1a* message in aging CD8$^+$T$_M$ was noteworthy [9] in light of earlier work with SAP$^{-/-}$ mice that demonstrated enhanced I^o virus-specific CD8$^+$T$_E$ activity [68], presumably due to an impairment of activation-induced cell death (AICD) [69]. In our experiments, however, expression of SAP protein

by aging CD8$^+$T$_M$ did not decline [9], and eight days after mixed AT/RC Arm, the activation-induced increase of SAP was comparable between young and old II^o CD8$^+$T$_E$ (not shown).

Nevertheless, we chose to explore the additional possibility of differential SAP induction specifically in the earliest phase of the II^o response. To this end, we employed the p14 chimera model and compared the initial recall response of young and aged p14 T$_M$ by CFSE dilution *in vivo* and *in vitro*. Although we observed similar proliferation patterns for all II^o p14 T$_E$ populations (Fig 6A), more detailed analyses of the *in vitro* studies suggested that aged p14 T$_M$ might start to divide a little earlier (*i.e.*, exhibiting a ~1.4-fold higher division indices) yet the identical proliferation indices of young and old II^o p14 T$_E$ (Fig 6A) are consistent with our earlier conclusion about the comparable antigen-driven proliferation of peripheral young and old II^o CD8$^+$T$_E$ [9]. Importantly though, the better survival of aged II^o CD8$^+$T$_E$ in our *in vivo* model [9] corresponded to higher numbers of old II^o p14 T$_E$ surviving in the *in vitro* culture system (Fig 6A) supporting the general utility of the latter experimental approach. We then proceeded with the quantification of SAP expression as a function of *in vitro* proliferation and found that the early II^o effector phase of young but not old p14 T$_M$ was accompanied by a significant elevation of SAP levels (Fig 6A). Thus, the increased *in vitro* accumulation of aged II^o p14 T$_E$ correlates with their lower SAP expression which is consistent with the notion of impaired AICD in the absence of SAP [69].

To formally evaluate the hypothesis that the amount of induced SAP expression determines the recall reactivity of II^o CD8$^+$T$_E$ populations, we generated retroviral p14 chimeras that overexpress SAP selectively in subpopulations of p14 T$_{E/M}$ as detailed in Fig 6B/6C and Methods. Following purification of p14 T$_M$ transduced with SAP or control retroviruses, AT into naïve B6 hosts and re-challenge with LCMV Arm or cl13 (Fig 6D), enforced SAP expression indeed compromised II^o p14 T$_E$ expansions (Fig 6E/6F and not shown). Collectively, our experiments therefore indicate that the improved antigen-driven II^o expansion of aged CD8$^+$T$_M$ is facilitated by their restrained upregulation of SAP expression. In the chronic LCMV model, the SLAMF4/CD244 receptor was recently assigned a predominantly inhibitory function as based on enhanced NK cell activity in CD244$^{-/-}$ mice as well as greater II^o reactivity of CD244$^{-/-}$ p14 T$_M$ in the AT/RC cl13 system [66, 70], and most recent work specifies that inhibitory functions exerted by the entire *Slam* locus on NK cell responses are solely based on CD244 activity [71]. It should therefore be interesting to assess if the early recall response of CD244$^{-/-}$ CD8$^+$T$_M$ also involves a subdued induction of SAP.

Discussion

In contrast to the wealth of information detailing the roles of multiple TCR-independent determinants in shaping I^o pathogen-specific CD8$^+$T$_E$ responses, considerably less information is available about the regulation of II^o CD8$^+$T$_E$ immunity [72, 73]. Moreover, the notion of long-term CD8$^+$T cell memory as a progressively evolving trait has gained increasing traction over the past half decade and implies the potential remodeling of relevant recall response determinants; to date, however, their identities and possible contributions to altered II^o CD8$^+$T$_E$ reactivity remain largely unknown. To begin to address these issues, we took advantage of a recent screen that correlates a surprising abundance of distinctive molecular, phenotypic and functional properties of aged CD8$^+$T$_M$ populations with their enhanced recall capacity [9], and interrogated the specific contribution of 15 molecular pathways to the regulation of II^o CD8$^+$T$_E$ expansions. Our results are noteworthy for the identification of 1., diverse molecular interactions that embellish II^o CD8$^+$T$_E$ responses in general and those of old CD8$^+$T$_M$ in particular; 2., an age-dependent convergence between an acquisition of naïve-like CD8$^+$T$_M$ properties [9] and an enhanced reliance on recall response determinants critical for

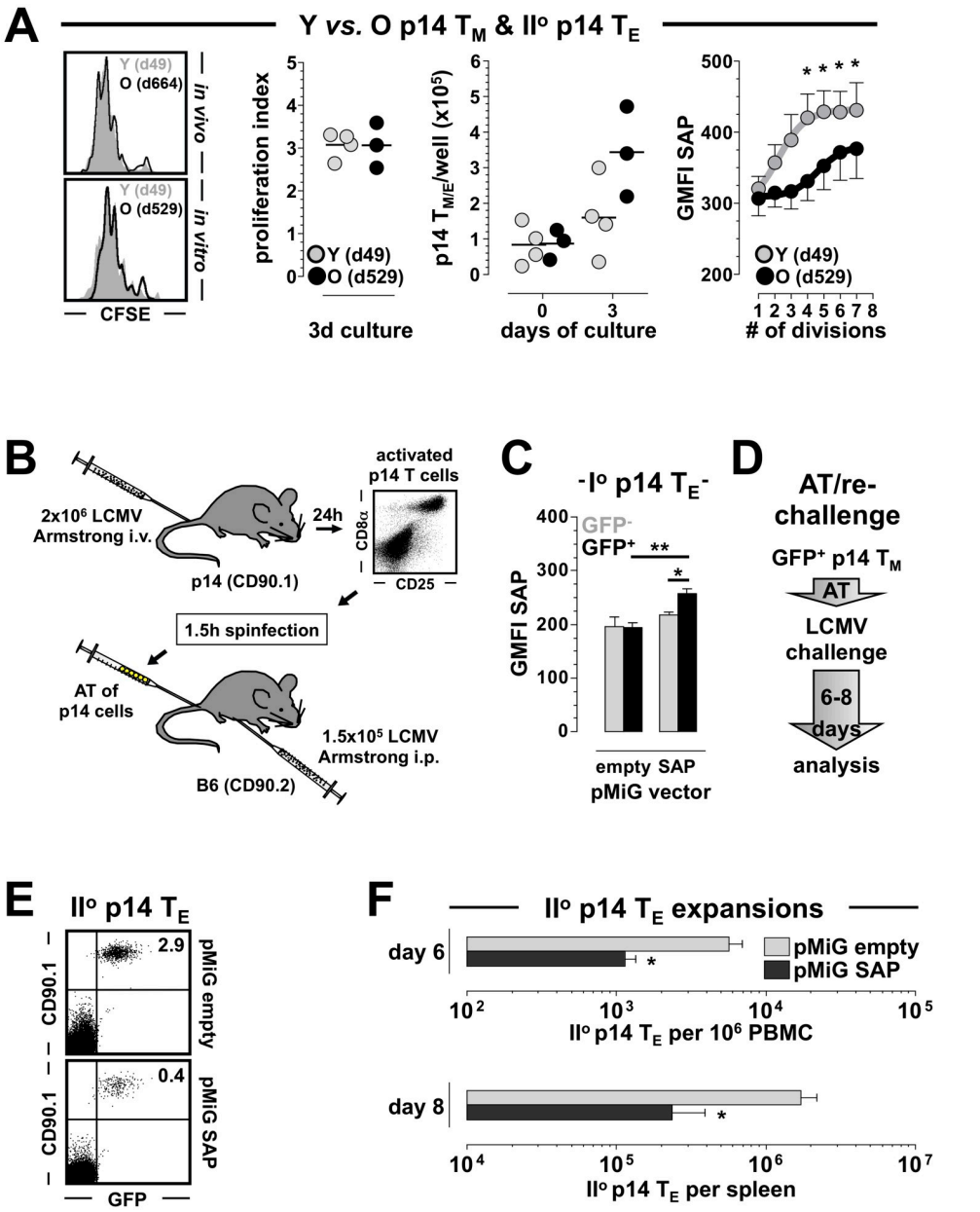

Fig 6. Enforced SAP expression constrains II^{o} reactivity of CD8^{+}T$_{M}$. A., proliferation of young and old II^{o} p14 T$_{E}$ as determined by CFSE dilution *in vivo* (64h after AT/RC Arm with 10^{6} p14 T$_{M}$) and *in vitro* (PBMC containing equal numbers of p14 T$_{M}$ cultured for 72h with GP$_{33–41}$ peptide-coated APCs). The adjacent diagrams depict *in vitro* proliferation indices, absolute numbers of p14 T cells at start (d0) and end (d3) of culture, and SAP expression as a function of cell division. **B.,** flow chart for construction of retrogenic p14 chimeras including 1.5h *in vitro* spinfection with pMiG-empty (control) or pMiG-SAP (experimental) retroviruses. **C.,** total SAP content of I^{o} p14 T$_{E}$ (d8) comparing control and experimental p14 chimeras as well as transduced (GFP^{+}) and untransduced (GFP^{-}) subsets. **D.,** experimental flow chart: GFP^{+} p14 T$_{M}$ (d89) were FACS-purified from control and experimental p14 chimeras and transferred into B6 mice ($2x10^{3}$/recipient) that were then challenged with LCMV Arm and analyzed 6–8 days later. **E.,** II^{o} p14 T$_{E}$ expansions in peripheral blood (d6); dot plots gated on all PBMC, note that GFP expression is restricted to the transferred congenic CD90.1^{+}p14 T cells. **F.,** summary of II^{o} p14 T$_{E}$ expansions in blood (d6) and spleen (d8); n≥3 mice/group.

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Table 1. Recall response determinants for aging CD8$^+$T$_M$ populations: Summary of experimental interventions and outcomes.

Pathway	AT/RC Intervention	Effect on II^o CD8$^+$T$_E$ expansions: AT/RC Arm	Effect on II^o CD8$^+$T$_E$ expansions: AT/RC cl13
IL-4: IL-4R	IL-4$^{-/-}$ recipients	O ~ Y	**O > Y**
IL-6: IL-6R	IL-6$^{-/-}$ recipients	O ~ Y	O ≥ Y
IL-7: IL-7R	IL-7/IL-7R blockade	O ~ Y	O ~ Y
TSLP: IL-7R	IL-7/IL-7R blockade	O ~ Y	O ~ Y
IL-15: IL-15R	IL-15$^{-/-}$ recipients	O > Y*	n.d.
TGFβ: TGFbR	TGFβ blockade	O ~ Y	O ~ Y
IFNγ: IFNγR	IFN$\gamma^{-/-}$ recipients	O < Y	n.d.
	IFNγ blockade	O < Y**	**O ~ Y****
Fas: FasL	B6.lpr recipients	O ≥ Y	**O > Y**
CD40L: CD40	CD40L blockade	O ~ Y	O < Y
CD4$^+$T cell help	CD4$^+$T cell depletion	n.d.	O < Y
LFA-1: ICAM1/2	LFA-1 blockade	O ≥ Y	**O > Y**
CD27: CD70	CD70 blockade	O ~ Y	O ~ Y
CD28: CD80/86	CD28 blockade	**O > Y**	n.d.
	CD80/86$^{-/-}$ recipients	O ~ Y	**O > Y**
CXCR3: CXCL9/10	CXCR3 blockade	**O > Y**	n.d.
SLAMR: SAP	SAP overexpression	**SAP^{OE} > SAP^{ctrl}**	**SAP^{OE} > SAP^{ctrl}**

CD8$^+$T$_M$ recall responses were quantified using an experimental adoptive transfer/re-challenge (AT/RC) approach under conditions of systemic antibody blockade, particular immunodeficiency of CD8$^+$T$_M$ recipients, or targeted gene overexpression by transferred CD8$^+$T$_M$; the Table specifically summarizes the effects of the various experimental interventions on the relative extent of old vs. young II^o CD8$^+$T$_E$ expansions in comparison to respective control cohorts. O and Y: old and young CD8$^+$T$_M$; n.d.: not determined; SAPOE and SAPctrl: SAP-overexpressing and control CD8$^+$T$_M$.

*IL-15-deficiency of recipients is the only experimental condition that increased rather than reduced old (but not young) II^o CD8$^+$T$_E$ expansions.

**While IFNg production by host cells preferentially promotes young II^o CD8$^+$T$_E$ expansions, a greater importance of CD8$^+$T$_M$-produced IFN? for old II^o CD8$^+$T$_E$ expansions can be deduced from the direct comparison of II^o expansions in IFNg$^{-/-}$ recipients and under conditions of systemic IFNg blockade (cf., Fig 3B/3C).

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the effective priming of naïve CD8$^+$T cells; 3., the distinct outcomes observed after acute *vs.* chronic viral re-challenge; and 4., the receptors/ligands that, against expectation, did not participate in control of II^o CD8$^+$T$_E$ reactivity (Table 1). Collectively, our observations reveal a previously unappreciated temporal contingency of recall responses that endows aged CD8$^+$T$_M$ with the capacity to more effectively harness a multiplicity of disparate molecular interactions in the wake of a re-infection. Accordingly, our emphasis of mechanistic diversity over detail in the present study may also serve as an outline and orientation for future in-depth investigations into specific molecular pathways that are of relevance to the regulation of II^o CD8$^+$T$_E$ immunity.

Here, we focused our attention on selected pathways comprising cytokine signaling, T cell trafficking, co-stimulation and -inhibition, and effector functionalities that might constitute pertinent recall response determinants for old CD8$^+$T$_M$ on account of the long-term expression kinetics previously reported for the respective CD8$^+$T$_M$-expressed receptors/ligands (the relative robustness of these temporal expression patterns is now supported by an extension of our earlier analyses to aging antiviral CD8$^+$T$_M$ populations in the blood, and to subsets with different epitope specificities and TCR affinities/avidities [9]). For consistency and comparative purposes, we employed a mixed AT/RC setting where the II^o expansions of the dominant D^{b}NP$_{396}^+$ CD8$^+$T$_E$ population peak on d8 after re-challenge [9]; other epitope-specific CD8$^+$T$_E$ populations may display slightly different recall kinetics, but we emphasize that

expansion differences captured at different time points during the II^o CD8$^+$T$_E$ stage largely carry over into the II^o CD8$^+$T$_M$ phase and thus reflect not only a differential recall but also a distinct II^o memory potential of CD8$^+$T$_M$ subsets with disparate phenotypic/functional properties (ref.[9] and S3B–S3D Fig). While our choice of II^o CD8$^+$T$_E$ expansions as a principal analytical modality is based on its direct correlation with immune protection [9], we note that the precise virus clearance kinetics in our model system are contingent on multiple variables (maturation stage and number of separately transferred CD8$^+$T$_M$, nature of the II^o challenge, type of experimental pathway blockade) such that differential virus control may only be observed in certain "combinatorial scenarios" as shown previously [66]. In contrast, the quantification of divergent II^o CD8$^+$T$_E$ expansions, especially those derived from two distinct CD8$^+$T$_M$ populations within the same host, is a more robust measure that is, within limits, impervious to the number of transferred CD8$^+$T$_M$ [9]. Accordingly, a detailed interrogation of II^o CD8$^+$T$_E$ kinetics, associated virus suppression and II^o CD8$^+$T$_M$ development is beyond the scope of the present work and will be reserved for our future investigations into selected mechanisms operative in the regulation of CD8$^+$T$_M$ recall responses.

With these caveats in mind, our findings collectively indicate that IL-4-, LFA-1-, CXCR3- and CD28-dependent interactions, restrained induction of SAP expression, as well as CD8$^+$T$_M$-produced FasL and IFNγ not only promote overall enhanced II^o CD8$^+$T$_E$ expansions, but in particular convey a set of heterogeneous signals that collectively boost the recall reactivity of aged CD8$^+$T$_M$ populations (Table 1). While the relative contribution of individual molecular pathways to the regulation of recall responses ranges from the modest to the more pronounced (*e.g.*, a 2.8-fold reduction of LCMV Arm-driven aged II^o CD8$^+$T$_E$ expansions under conditions of IFNγ neutralization *vs.* an up to 13-fold inhibition in the context of CD28 blockade), the overall efficacy of II^o CD8$^+$T$_E$ immunity and immune protection [9] is shaped by the integrated activity of different pathways the individual or combined therapeutic targeting of which may in fact allow for the tailored modulation of specific CD8$^+$T$_M$ responses. As a proof-of-principle for the latter contention, we show in additional experiments that combined CD28 and CXCR3 blockade exerts a synergistic effect by compromising young and especially old CD8$^+$T$_M$ recall responses to an extent that substantially exceeds the effects of individual pathway blockade (S4 Fig).

Furthermore, the above molecular interactions mostly are of greater importance for the regulation of II^o CD8$^+$T$_E$ immunity in response to chronic rather than acute viral challenges. Recent work supports the notion that the eventual or at least partial control of chronic viral infections relies on a multiplicity of specific determinants that are often dispensable for the clearance of acute virus infections [74]. Our observations extend this concept to the context of II^o CD8$^+$T$_E$ responses by documenting that CD8$^+$T$_M$, far from being "co-stimulation independent", also require the productive engagement of diverse molecular pathways to unfold their full recall potential when confronted with a chronic virus challenge. A further elucidation of these phenomena might very well help to establish an adjusted perspective onto one of the central tenets of T cell memory, namely its presumed imperviousness to the modulation by biochemical pathways commonly referred to as "signal 2 & signal 3" [75]. In fact, the "rebound model" [9], together with the present report, suggests that aging CD8$^+$T$_M$ become increasingly reliant on the very same "signal 2 & signal 3" interactions that, dependent on the experimental system, also control I^o CD8$^+$T$_E$ differentiation.

Two pathways interrogated in the present study were found to be of preferential importance to the regulation of young rather than old II^o CD8$^+$T$_E$ responses (Table 1). Here, the greater dependence of young CD8$^+$T$_M$ recall responses on CD4$^+$T cell help and CD40L-mediated interactions in the chronic LCMV system is essentially consistent with the "rebound model", but the enhanced reliance of young CD8$^+$T$_M$ on non-CD8$^+$T$_M$-produced IFNγ,

despite reduced CD119/IFNγR1 expression and sensitivity, was unexpected. IFNγ can exert both stimulatory and inhibitory effects on CD8$^+$T$_E$ populations [30, 76], and the specific balance achieved between these opposing signals may be distinct for young and old CD8$^+$T$_M$, perhaps as a result of differential IFNγR2 induction [77], but ultimately the reasons for the greater role of host IFNγ in control of young II^o CD8$^+$T$_E$ immunity remain unclear. We also found that several other cytokine signaling and co-stimulatory pathways (IL-6, IL-7, IL-15, TSLP, TGFβ, CD27:CD70) appeared to have at best a minor impact on the regulation of II^o CD8$^+$T$_E$ responses (Table 1). While these results underscore the obvious fact that promising clues gleaned from a comprehensive set of databases [9] need not necessarily translate into biologically relevant differences within a given model system, they neither rule out potential redundancies not investigated in the present study nor the possibility that these as well as additional pathways may be operative in the context of other experimental and naturally occurring scenarios. Therefore, in as much as the "rebound model" of extended CD8$^+$T$_M$ maturation applies to pathogen infections in general, the progressive "de-differentiation" of aging CD8$^+$T$_M$, especially given the "programmed" nature of this process [9], may allow them to brace for more effective recall responses under a greater variety of productive pathogen re-encounters. At the same time, the multitude of diverse molecular pathways involved in shaping improved clinical outcomes also provides an abundance of different targets for potential therapeutic interventions.

Materials and methods

Ethics statement

All procedures involving laboratory animals were conducted in accordance with recommendations issued in the "Guide for the Care and Use of Laboratory Animals of the National Institutes of Health", the protocols were approved by the Institutional Animal Care and Use Committees (IACUC) of the University of Colorado (70205604[05]1F, 70205607[05]4F and B-70210[05]1E) and Icahn School of Medicine at Mount Sinai (IACUC-2014-0170), and all efforts were made to minimize suffering of animals.

Mice, virus and challenge protocols

C57BL6/J (B6), congenic B6.CD90.1 (B6.PL-*Thy1^a*/CyJ) and B6.CD45.1 (B6.SJL-*Ptprc^a Pepc^b*/BoyJ), IL-4$^{-/-}$ (B6.129P2-*Il4^{tm1Cgn}*/J), IL-6$^{-/-}$ (B6.129S2-*Il6^{tm1Kopf}*/J), IFN$\gamma^{-/-}$ (B6.129S7-*Ifng^{tm1Ts}*/J), CD80/86$^{-/-}$ (B6.129S4-*Cd80^{tm1Shr}Cd86^{tm2Shr}*/J) and lpr (B6.MRL-*Fas^{lpr}*/J) mice on the B6 background were purchased from The Jackson Laboratory; B6.IL-15$^{-/-}$ (C57BL/6NTac-*IL15^{tm1Imx}* N5) mice were acquired from Taconic; and p14 mice harboring TCRtg CD8$^+$T cells specific for the dominant D^b-restricted LCMV-GP$_{33-41}$ determinant were obtained on a B6.CD90.1 background from Dr. M. Oldstone. We only used male mice in this study to avoid potential artifacts that may arise in gender mismatched AT settings. LCMV Armstrong (clone 53b) and clone 13 (cl13) were obtained from Dr. M. Oldstone, and grown and titered as described [78]. For I^o challenges, 8–10 week old mice were infected with a single intraperitoneal (i.p.) dose of 2x10^5 plaque-forming units (pfu) LCMV Arm, housed under SPF conditions and monitored for up to ~2 years; for II^o challenges, naïve congenic recipients of various CD8$^+$T$_M$ populations were inoculated with 2x10^5 pfu LCMV Arm i.p. or 2x10^6 pfu LCMV cl13 i.v. as discussed elsewhere [9, 10], exclusion criteria for aging LCMV-immune mice in this study included gross physical abnormalities (lesions, emaciation and/or pronounced weight loss), lymphatic tumors as indicated by enlarged LNs at time of necropsy, and T cell clonal expansions among virus-specific CD8$^+$T$_M$ populations (D^{b}NP$_{396}^+$, D^{b}GP$_{33}^+$ or

$D^bGP_{276}^+$); according to these criteria, up to ~30% of aging mice were excluded from the study.

Tissue processing, cell purification and adoptive transfers (AT)

Lymphocytes were obtained from blood, spleen and LNs according to standard procedures [79]. Enrichment of splenic T cells was performed with magnetic beads using variations and adaptations of established protocols [9] and reagents purchased from StemCell Technologies, Miltenyi Biotec and Invitrogen/Caltag/Thermofisher (S1 Table). For mixed AT/RC experiments, $CD8^+T_M$ from young and aged LCMV-immune B6 and B6-congenic donors were enriched by combined depletion of B and $CD4^+$T cells (or only B cells) followed by 1:1 combination at the level of $D^bNP_{396}^+CD8^+T_M$, i.v. AT of mixed populations containing 2–10x10^3 young and old $D^bNP_{396}^+CD8^+T_M$ each into naïve congenic recipients, and challenge with LCMV (Figs 1A–1D, 2C–2E, 3B–3D, 4C/4E & 5B–5F). For construction of p14 chimeras [9], $CD8^+$T cells were enriched from spleens of naïve $CD90.1^+$ p14 mice by negative selection, and 5x10^4 purified p14 cells were transferred i.v. into B6 recipients prior to LCMV infection 2–24h later. We note that p14 T_M differentiation in this model system is contingent on p14 T_N input numbers [80, 81] an increase of which will in fact accelerate p14 T_M maturation kinetics *at large* [9]. While chimera generation with 5x10^4 p14 T_N may therefore underestimate differences between young and aged p14 T_M, our use of this model is restricted here to an effective demonstration of their differential cytokine responsiveness (Figs 2B & 3A), new analyses of previously generated data sets [9] (Fig 4A & S1 Fig), and the revelation of distinctive functional properties during the earliest stages of the young *vs*. old p14 T_M recall response (Fig 6A). In the latter experiments, 10^6 young or old CFSE-labeled p14 T_M were transferred into B6 recipients that were then challenged with LCMV Arm, and *in vivo* proliferation of II^o p14T_E was analyzed 64h later as detailed in ref.[9] (Fig 6A).

Stimulation cultures

Splenic single cell suspensions prepared from young and old LCMV-immune p14 chimeras were cultured for 15min in complete RPMI with graded dosages of recombinant cytokines (murine IL-4, IL-6, IL-10, IFNγ; Peprotech) prior to fixation with PFA buffer, processing and combined CD90.1 and intracellular pSTAT staining (Figs 2B & 3A). For *in vitro* proliferation and survival assays, lympholyte-purified PBMC from young and old LCMV-immune p14 chimeras were labeled with CFSE, adjusted to contain the same number of p14 T_M, and cultured for 72h with T cell-depleted, LCMV-$GP_{33–41}$ peptide-coated B6 spleen cells; numbers of surviving p14 T cells were subsequently calculated using Countess (Invitrogen) or Vi-Cell (Beckmann Coulter) automated cell counters (Fig 6A).

Flow cytometry

All reagents and materials used for analytical flow cytometry are summarized in S1 Table, and our basic staining protocols are described and/or referenced in ref.[9]. Detection of phosphorylated signal transducer and activator of transcription (STAT) proteins (Figs 2B & 3A) was performed using methanol-based cell permeabilization as described [82]. All samples were acquired on FACSCalibur, LSR II (BDBiosciences), Cyan (Beckman Coulter) or Attune NxT (Thermofisher) flow cytometers and analyzed with DIVA (BDBiosciences) and/or FlowJo (TreeStar) software; and visualization of *in vivo* and *in vitro* T cell proliferation by step-wise dilution of CFSE as well as calculation of proliferation and division indices was performed using the FlowJo "proliferation platform" (Fig 6A). To isolate $CD8^+T_M$ subsets with differential CXCR3 expression levels (S3A Fig), live (Zombie $Violet^-$) $CXCR3^{hi}$ and $CXCR3^{lo}$

populations were sorted on a FACSAria (BDBiosciences) from spleen cells obtained from LCMV-immune B6.CD90.1 mice and pre-enriched by magnetic bead depletion of CD4$^+$T and CD19$^+$B cells (Miltenyi).

Microarray analyses

Details for microarray analyses of highly purified p14 $T_{E/M}$ populations and time series GSEAs (Fig 4A & S1 Fig) are found in refs.[9, 10]; all data can be retrieved from the GEO repository (accession number GSE38462; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE38462).

In vivo antibody treatment

For *in vivo* blockade of cytokine signaling, T cell trafficking or co-stimulation (Figs 1B/1C, 2E, 3B/3C, 4C/4E & 5B–5D/5F), naïve B6 or B6 congenic recipients were injected i.p. with antibodies ~2h before AT of mixed CD8$^+$$T_M$ populations and subsequent LCMV infection as well as on d2 and d4 after challenge (αIL-7 [M25] & αIL-7Ra [A7R34]: 3x500μg each; αTGFβ$_{1,2,3}$ [1D11.16.8]: 3x1000μg; αIFNγ [XMG1.2]: 3x1000μg; αCXCR3 [CXCR3–173]: 3x100μg; αCD70: [FR70]: 3x250μg; αCD154/CD40L [MR1]: 3x250μg; αCD28 [37.51]: 3x100μg; αCD62L [MEL-14]: 3x150μg); αCD11a/LFA-1 [KBA]: 2x200μg on d0 and d2 only; corresponding control antibodies: dosages commensurate to experimental antibodies; for combination treatment [S4 Fig], the same schedule was employed using 3x200μg hamster IgG, 3x100μg αCD28, 3x100μg αCXCR3, or 3x100μg αCD28 and αCXCR3 each); *in vivo* CD4$^+$T cell depletion was achieved by i.p. injection of 200μg GK1.5 antibody on days -1 and +1 in relation to AT/RC [83]. Further details about all *in vivo* antibodies are provided in S1 Table.

Retroviral transductions, chimera construction and transduced p14 T_M purification

Murine *Sh2d1a* (SAP) cDNA was purchased from Open Biosystems (clone ID 1400188) and sub-cloned into a murine stem cell virus- (MSCV-) based retroviral pMiG vector that contains green fluorescent protein (GFP) as a reporter (gift from Dr. P. Marrack). To generate retroviruses, pMiG-empty or pMiG-SAP plasmids were co-transfected with PsiEco helper plasmid into Phoenix 293T cells using Fugene 6 (Roche) according to standard procedures [82]. After 48h, retroviral supernatants were harvested and spin-transductions of *in vivo* activated p14 splenocytes (naïve p14 mice infected with 2x10^6 pfu LCMV Arm i.v. 24h earlier) were performed for 90min at 32˚C in the presence of 8μg/mL polybrene, 10mM HEPES and 10μg/mL recombinant hIL-2. Transduced p14 splenocytes were transferred "blind" into naïve B6 mice that were subsequently infected with 2x10^5 pfu LCMV Arm i.p. (Fig 6B), and effective transduction levels were verified in blood-borne p14 T_E 8 days later (Fig 6C). For subsequent AT/RC experiments, transduced p14 T_M (CD4$^-$B220$^-$CD90.1$^+$GFP$^+$) were purified from spleens using a Coulter Moflo XDP cell sorter.

Statistical analyses

Data handling, analysis and graphic representation was performed using Prism 6.0c (GraphPad Software). All data summarized in bar and line diagrams are expressed as mean ±1 standard error (SEM), and asterisks indicate statistical differences calculated by Student's t-test or one-way ANOVA with Dunnett's multiple comparisons test, and adopt the following convention: *: $p<0.05$, **: $p<0.01$ and ***: $p<0.001$.

Supporting information

S1 Fig. Gene set enrichment analysis (GSEA) of aging p14 T_M: The JAK-STAT signaling pathway. Time series GSEA were conducted with data sets obtained for aging p14 T_M (d46, d156, d286 and d400) purified from LCMV-challenged p14 chimeras and processed directly *ex vivo* for microarray hybridization as detailed in refs.[9, 10]. Top: aged p14 T_M are enriched for genes within the KEGG JAK-STAT signaling pathway module (normalized enrichment score: 1.05). Bottom: heat map displaying relative expression of individual genes by aging p14 T_M (blue: low, red: high). The right hand column summarizes corresponding protein expression patterns conducted with aging $D^bNP_{396}^+$ and/or $D^bGP_{33}^+$ CD8$^+$$T_M$ retrieved from spleen or blood of LCMV-immune B6 mice; colors identify significant expression changes accrued over time (red: upregulation; black: no change; green: downregulation); where indicated in parenthesis, CD8$^+$$T_M$ were stimulated *in vitro* prior to analysis (IFNγ: 5h TCR stimulation with peptide; phosphorylated STAT proteins: 15min stimulation with indicated cytokines). The primary protein expression data in this summary are shown in Figs 2A/2B, 3A, S2 Fig and/or refs.[9, 10].
(TIF)

S2 Fig. Temporal regulation of selected CD8$^+$$T_M$-expressed cell surface receptors/ligands. PBMC obtained from cohorts of aging LCMV-immune mice were contemporaneously stained to quantify expression levels of indicated receptors/ligands by $D^bGP_{33}^+$ CD8$^+$$T_M$ (GMFI: gometric mean of fluoresecence intensity; n$\geq$3 mice per time point; statistical differences between young and older CD8$^+$$T_M$ were calculated using one-way ANOVA with Dunnett's multiple comparisons test).
(TIF)

S3 Fig. Differential recall and II^o memory potential of young CXCR3^{hi} *vs*. CXCR3^{lo} CD8$^+$$T_M$ subsets. CXCR3^{hi} and CXCR3^{lo} CD8$^+$T cell subsets were purified from young LCMV-immune B6.CD90.1 donors (d46) by combined magnetic bead and fluorescence activated cell sorting, and transferred i.v. into separate B6 recipients that were subsequently challenged with LCMV Arm (CXCR3^{hi} transfers contained 5.0x10^3 $D^bNP_{396}^+$ and 1.2x10^3 $D^bGP_{33}^+$ CD8$^+$$T_M$ per recipient, CXCR3^{lo} transfers contained 5.0x10^3 $D^bNP_{396}^+$ and 1.35x10^3 $D^bGP_{33}^+$ CD8$^+$$T_M$ per recipient). **A.,** identification of $D^bNP_{396}^+$ and $D^bGP_{33}^+$ CD8$^+$$T_M$ (left) and representative CXCR3 (and CX3CR1) expression pattern by $D^bNP_{396}^+$ and $D^bGP_{33}^+$ CD8$^+$$T_M$ (right; the indicated regions demarcate CXCR3^{hi} and CXCR3^{lo} subsets corresponding to our sorting strategy). Values in dot plots are the percentage of cells within the indicated regions. **B.,** kinetics of II^o $D^bNP_{396}^+$ (left) and $D^bGP_{33}^+$ (right) CD8$^+$$T_E$ expansions as well as II^o CD8$^+$$T_M$ development in peripheral blood (black: II^o CD8$^+$$T_{E/M}$ derived from CXCR3^{hi} donor CD8$^+$$T_M$, gray: II^o CD8$^+$$T_{E/M}$ derived from CXCR3^{lo} donor CD8$^+$$T_M$). **C.,** specific II^o CD8$^+$$T_M$ abundance in spleen (d30 after AT/RC); n = 5 mice per group and time point in panels B and C. **D.,** histograms are gated on I^o (donor, d46) or II^o (d46 + d30) $D^bNP_{396}^+$ CD8$^+$$T_M$ as indicated; the respective tracings correspond to the phenotypes of either CXCR3^{lo} (gray filled) or CXCR3^{hi} (black) I^o donor CD8$^+$$T_M$ subsets, or to those of II^o CD8$^+$$T_M$ derived from CXCR3^{lo} (gray) *vs*. CXCR3^{hi} (black) I^o CD8$^+$$T_M$ (concatenated files of three mice/group; values featured in black or gray are the percentage of respective I^o or II^o CD8$^+$$T_M$ expressing high levels of indicated cell surface antigens). Note that CXCR3^{hi}-derived II^o CD8$^+$$T_M$ display a more mature phenotype as indicated by partial re-expression of high CXCR3, CD27 and CD127 levels as well as downregulation of CD43 (115kd glycoform), CX3CR1 and in particular KLRG1 (no differences were noted for CD62L and CD122 expression).
(TIF)

S4 Fig. Synergy of combined CD28 and CXCR3 blockade. Mixed AT/RC Arm experiments were conducted under conditions of separate or combined CD28/CXCR3 blockade, and the bar diagram enumerates II^o D^{b}GP$_{33}$$^+$ CD8$^+$T$_E$ in the spleen with arrows, values and associated asterisks (significance) indicating the factors by which indicated treatment modalities reduced respective old and young recall responses in comparison to hamster IgG-treated control mice. While the overall reduction of II^o CD8$^+$T$_E$ expansions after individual pathway blockade was somewhat less (αCD28 treatment) or more (αCXCR3 treatment) pronounced than in our other experiments (also evident at the level of impaired I^o CD8$^+$T$_E$ responses), combined CD28/CXCR3 blockade decreased old CD8$^+$T$_M$ recall responses to a significantly greater extent than CD28 or CXCR3 blockade alone ($p = 0.0204$ and $p = 0.0015$, respectively); a similar synergism was also observed for the inhibition of young CD8$^+$T$_M$ recall responses (αCD28/CXCR3 *vs.* αCD28 treatment $p = 0.0022$). These differences began to emerge in peripheral blood as early as d5 after AT/RC where only combination treatment resulted in a significant attenuation of aged ($p<0.04$) and to a lesser extent also young ($p<0.04$) II^o CD8$^+$T$_E$ expansions (n = 3 mice/group and time point; AT of ~5x10^3 young and old D^{b}GP$_{33}$$^+$CD8$^+T_M$ each).
(TIF)

S1 Table. Reagents and materials. Details about all antibodies, staining dyes, magnetic beads, MHC-I monomers/tetramers, and recombinant cytokines used in the present study.
(PDF)

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