Immunity
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Bystander Chronic Infection Negatively Impacts Development of CD8$^+$ T Cell Memory

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http://dx.doi.org/10.1016/j.immuni.2014.04.010

SUMMARY

Epidemiological evidence suggests that chronic infections impair immune responses to unrelated pathogens and vaccines. The underlying mechanisms, however, are unclear and distinguishing effects on priming versus development of immunological memory has been challenging. We investigated whether bystander chronic infections impact differentiation of memory CD8$^+$ T cells, the hallmark of protective immunity against intracellular pathogens. Chronic bystander infections impaired development of memory CD8$^+$ T cells in several mouse models and humans. These effects were independent of initial priming and were associated with chronic inflammatory signatures. Chronic inflammation negatively impacted the number of bystander CD8$^+$ T cells and their memory development. Distinct underlying mechanisms of altered survival and differentiation were revealed with the latter regulated by the transcription factors T-bet and Blimp-1. Thus, exposure to prolonged bystander inflammation impairs the effector to memory transition. These data have relevance for immunity and vaccination during persisting infections and chronic inflammation.

INTRODUCTION

Chronic infections with persisting pathogens including hepatitis viruses, *Plasmodium*, mycobacteria, or intestinal helminths affect billions of people. In some settings persistent pathogens, such as herpes viruses or GB virus C, can provide intrinsic resistance to unrelated microbes (Barton et al., 2007; Vahidnia et al., 2012). However, accumulating epidemiological evidence suggests that many chronic infections can increase susceptibility and pathology induced by unrelated pathogens. For example, intestinal helminth infections enhance *Mycobacterium tuberculosis* (Mtb)-induced pulmonary pathology (Resende Co et al., 2007) and increase the risk of developing chronic disease upon hepatitis C virus (HCV) infection (Kamal et al., 2001). Mathematical models indicate that malaria actively contributes to the increased rate of infection with HIV (Abu-Raddad et al., 2006), suggesting that dysregulated immunity due to a bystander chronic infection might be responsible for the increased incidence of other infections. Immunity to vaccines is reduced in subjects with chronic infections, suggesting that bystander chronic infections can also negatively impact responses to vaccination (Cooper et al., 2001; Nookala et al., 2004; Elias et al., 2008). Nevertheless, our current understanding of bystander infections and coinfections is based largely on epidemiological data with limited insight into the immunological mechanisms and potential therapeutic strategies to overcome these effects.

Several stages of a developing immune response might be affected by bystander chronic infections (Stelekati and Wherry, 2012). For example, initial entry of microbes or uptake of vaccines might be impacted by an altered mucosal environment due to chronic infections (van Riet et al., 2007). Persisting *Schistosoma* (Bahgat et al., 2010), Mtb (Zhang et al., 1995), or *Plasmodium* (Hawkes et al., 2010) infections can enhance replication of unrelated pathogens, resulting in increased pathogen load in coinfected individuals. Intrinsic defects in innate immune cells, such as natural killer (NK) cells (Morishima et al., 2006) or dendritic cells (DCs) (van Riet et al., 2007) in chronically infected individuals might potentiate defects in early innate immune responses. In addition to early changes in pathogenesis and/or innate immunity, an altered cytokine milieu due to unrelated persisting infections can substantially skew effector T cell differentiation, proliferation, and effector function (Harcourt et al., 2005; van Riet et al., 2007; Moorman et al., 2011).

Altered vaccine-induced immunity in chronically infected individuals suggests that immunological memory might be affected by bystander chronic infections. Indeed, type I interferons (IFN-I) produced by bystander acute viral infections or administration of Toll-like receptor (TLR) agonists, have been implicated in the erosion of preexisting memory CD8$^+$ T cells (McNally et al., 2001; Bahl et al., 2006). However, this attrition of preexisting memory due to subsequent acute infections is not observed in

all settings (Vezys et al., 2009; Odumade et al., 2012) and the role of chronically sustained versus acutely induced IFN-I remains poorly understood. The global impact of different types of bystander chronic coinfections that might or might not induce IFN-I suggests that mechanisms other than IFN-I might also contribute to a dysregulation of memory $CD8^+$ T cell development.

Here, we examined how different bystander chronic infections affect $CD8^+$ T cell memory differentiation and defined common molecular pathways associated with altered development of immunological memory. Chronic bystander infections substantially impaired the development of memory $CD8^+$ T cells, independent of initial priming in several mouse models of persisting viral or parasitic infection, with similar changes in humans chronically infected with HCV. Moreover, these changes in memory $CD8^+$ T cell differentiation were associated with a transcriptional imprint of IFN-I-inducible genes but could occur in the absence of direct signaling through the IFN-α and IFN-β receptor (IFNAR) on $CD8^+$ T cells exposed to bystander chronic infection. Bystander chronic infection and inflammation had a substantial impact on $CD8^+$ T cell survival during the effector to memory transition. In addition, we identified the downstream transcriptional regulators T-box 21 (T-bet) and B lymphocyte induced maturation protein 1 (Blimp-1) as molecular mediators of the effect of bystander inflammation on differentiation, but not survival. These data revealed two distinct mechanisms underlying altered $CD8^+$ T cell memory during coinfection. These results also suggested that, while upregulation of IFN-I-inducible genes is a symptom of this bystander effect, additional transcriptional mechanisms allow redundancy between inflammatory pathways that cause skewing of $CD8^+$ T cell differentiation during bystander chronic infections. Therefore, modulating sustained inflammatory signaling during the crucial phase of effector to memory transitioning might provide therapeutic opportunities to restore optimal vaccine responses in patients with persisting infections or chronic inflammatory conditions.

RESULTS

Chronic Bystander Infections Impair the Transition of $CD8^+$ T Cells from Effector to Memory

To investigate the role of chronic bystander infection in memory $CD8^+$ T cell development, we used persistent lymphocytic choriomeningitis virus (LCMV) clone 13 infection in mice. Naive mice or mice chronically infected with LCMV clone 13 (at day 30 postinfection; d30 p.i.) were infected with *Listeria monocytogenes*-expressing OVA (LM-OVA), and Kb-$OVA_{257–264}$-specific $CD8^+$ T cell responses were examined 30 days later. Although the frequency of Kb-$OVA_{257–264}$-specific $CD8^+$ T cells was higher in LCMV-infected mice compared to controls, the absolute number of Kb-$OVA_{257–264}$-specific $CD8^+$ T cells was not increased (data not shown). However, expression of molecules associated with memory differentiation, such as CD62L, CD27, and CXCR3 was reduced on the OVA-specific $CD8^+$ T cells in LCMV-infected mice compared to controls (see Figure S1 available online). In addition, the proportion of memory precursor cells, defined as $CD127^+KLRG1^-$ cells, was decreased, suggesting altered memory differentiation in the presence of bystander chronic LCMV infection.

To circumvent complications of changes in pathogen replication and/or pathogenesis due to altered innate immunity and/or antigen-presenting cell function during direct coinfection, we used adoptive transfer approaches to specifically examine the impact of bystander chronic infection on the transition of effector $CD8^+$ T cells into the memory pool. CD45.2 TCR-transgenic, $OVA_{257–264}$-specific (OT1) $CD8^+$ T cells were primed in congenic CD45.1 wild-type (WT) C57BL/6 mice with vesicular stomatitis virus-expressing OVA (VSV-OVA). On d8 p.i., equal numbers of these OT1 effector $CD8^+$ T cells were adoptively transferred into recipient CD45.1 mice that were naive (control), infected with the acute strain of LCMV Armstrong (at d8 p.i.), or infected with the persistent strain of LCMV clone 13 (at d8 p.i.). After 30 days, substantially reduced numbers of memory OT1 $CD8^+$ T cells were detected in multiple tissues of recipient mice infected with LCMV clone 13 compared to either naive recipients or the LCMV Armstrong infected recipients (Figure 1A; data not shown). This effect was not due to differences in the "take" after adoptive transfer (Figure S2). In addition, the OT1 $CD8^+$ T cells in mice transitioning to chronic infection had lower expression of the memory-associated molecules CD127, CXCR3, CD27, and CD62L and contained an increased proportion of terminally differentiated $KLRG1^+CD127^-$ cells, as well as fewer cells in the long-lived $CD127^+KLRG1^-$ memory pool (Figure 1B). Moreover, interleukin-2 (IL-2) or tumor necrosis factor alpha (TNF-α) cytokine coproduction with IFN-γ was reduced, although IFN-γ production alone was not dramatically impacted (Figure 1B). Similar results were obtained using a bacterial (LM-OVA) instead of a viral (VSV-OVA) infection for priming of $CD8^+$ T cells (Figure S2). The effect of bystander chronic infection was also observed when the d8 effector OT1 cells were adoptively transferred to chronically infected recipient mice at d30 (Figures S3A and S3B) or at d120 of chronic LCMV infection (Figures S3C and S3D), though the magnitude of the impact of bystander chronic infection on the developing memory $CD8^+$ T cells declined as the severity of infection decreased over time. Thus, chronic bystander viral infection, in striking contrast to acutely resolved viral infection, impairs the efficient transition of $CD8^+$ T cells from effector to memory.

We next examined whether these observations could be extended to other chronic bystander infections that, for example, induce high amounts of IL-12, compared to the IFN-I biased LCMV infection. For this reason, we used the protozoan parasite *Toxoplasma gondii* (*T. gondii*). Effector (d8) OT1 $CD8^+$ T cells adoptively transferred into mice transitioning to a chronic infection with *T. gondii* (at d8 p.i.) exhibited altered memory CD8 T cell development, with reduced numbers of OT1 cells (Figure 2A). Bystander chronic *T. gondii* infection also impaired memory $CD8^+$ T cell differentiation, by reducing the expression of memory-associated molecules, increasing the proportion of terminally differentiated effector cells ($KLRG1^+CD127^-$) and significantly impairing cytokine production (Figure 2B). These data highlight a common effect of bystander chronic viral and parasitic infections and prolonged or chronic inflammation in substantially disrupting memory $CD8^+$ T cell development.

Because both LCMV and *T. gondii* induce sustained T helper 1 (Th1)-type inflammation, we further extended these

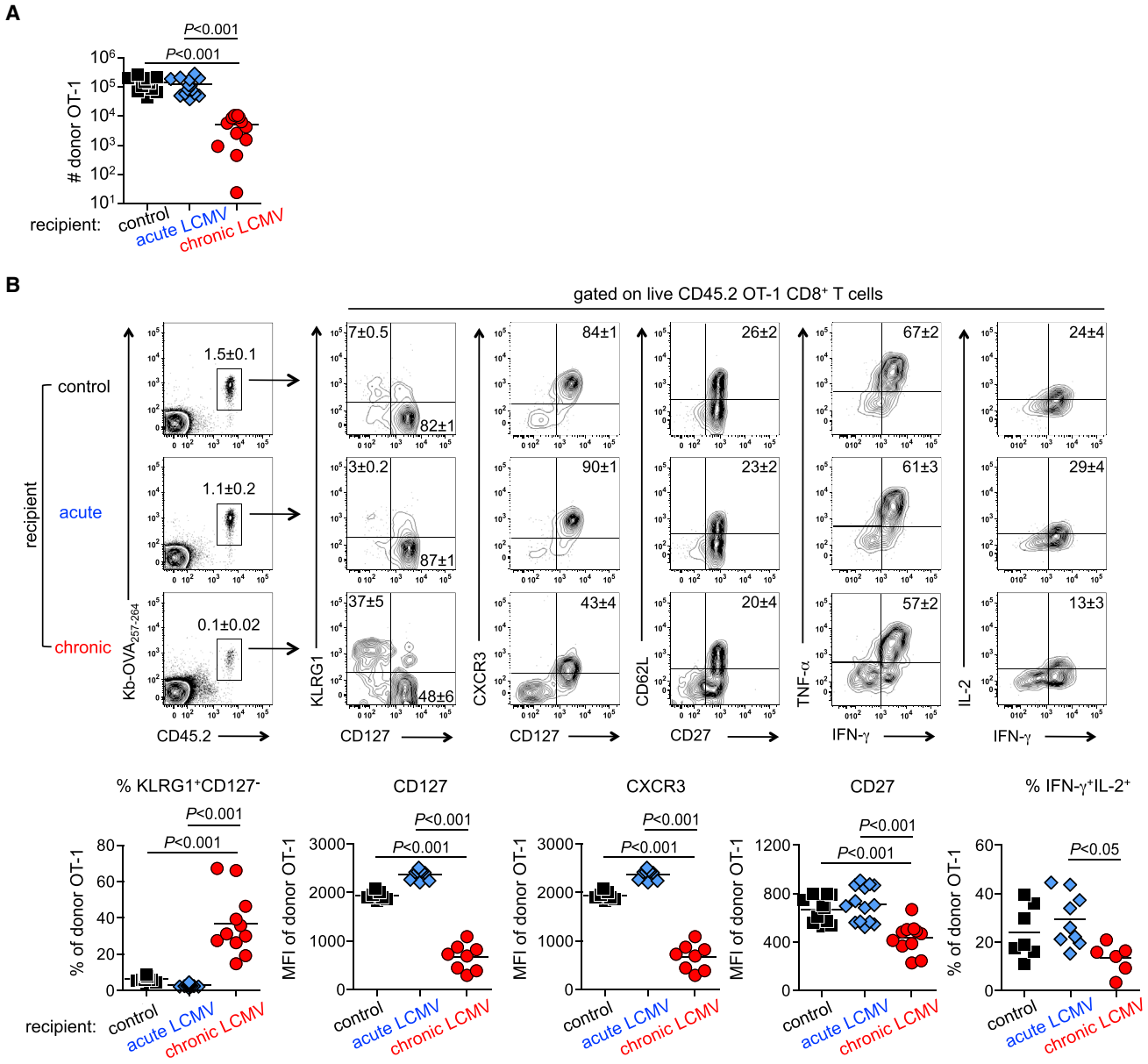

Figure 1. Bystander Chronic Viral Infection Disrupts the Effector to Memory Transition of CD8+ T Cells

CD45.2 OT1 CD8+ T cells were adoptively transferred to congenic CD45.1 mice. Recipients were infected with VSV-OVA. On d8 p.i., splenic CD45.2 OT1 CD8+ T cells were isolated and adoptively transferred to "control" (naive), "acute LCMV" (d8 post-LCMV Armstrong infection), or "chronic LCMV" (d8 post-LCMV clone 13 infection) recipient CD45.1 mice.

(A) OT1 cells were quantified in spleens 30 days posttransfer.

(B) Phenotype and cytokine production of OT1 cells were analyzed 30 days posttransfer in spleens (n > 9). Similar results were obtained from blood, liver, and bone marrow (BM; data not shown). Numbers on FACS plots indicate frequencies of respective populations ± SEM. Statistical significance was calculated with one-way ANOVA for normally distributed samples and Kruskal-Wallis test for nonequally distributed samples.

observations to a chronic helminth infection (*Heligmosomoides polygyrus*; *H. polygyrus*), inducing Th2-type inflammation. After adoptive transfer of effector (d8) OT1 CD8+ T cells, the numbers of memory OT1 cells recovered from mice chronically infected with *H. polygyrus* were reduced compared to control naive recipient mice (Figure S4A). Acquisition of key memory CD8+ T cell differentiation properties was also altered in the presence of bystander *H. polygyrus* infection, though not as dramatically as in the presence of chronic bystander LCMV or *T. gondii* infection (Figure S4B). Together, these data indicated an effect of bystander chronic viral or parasitic infections that induce different inflammatory environments, on the transition of effector CD8+ T cells into the memory pool. Disruption of these key differentiation events resulted in failure to appropriately undergo phenotypic and functional maturation associated with the effector to memory transition.

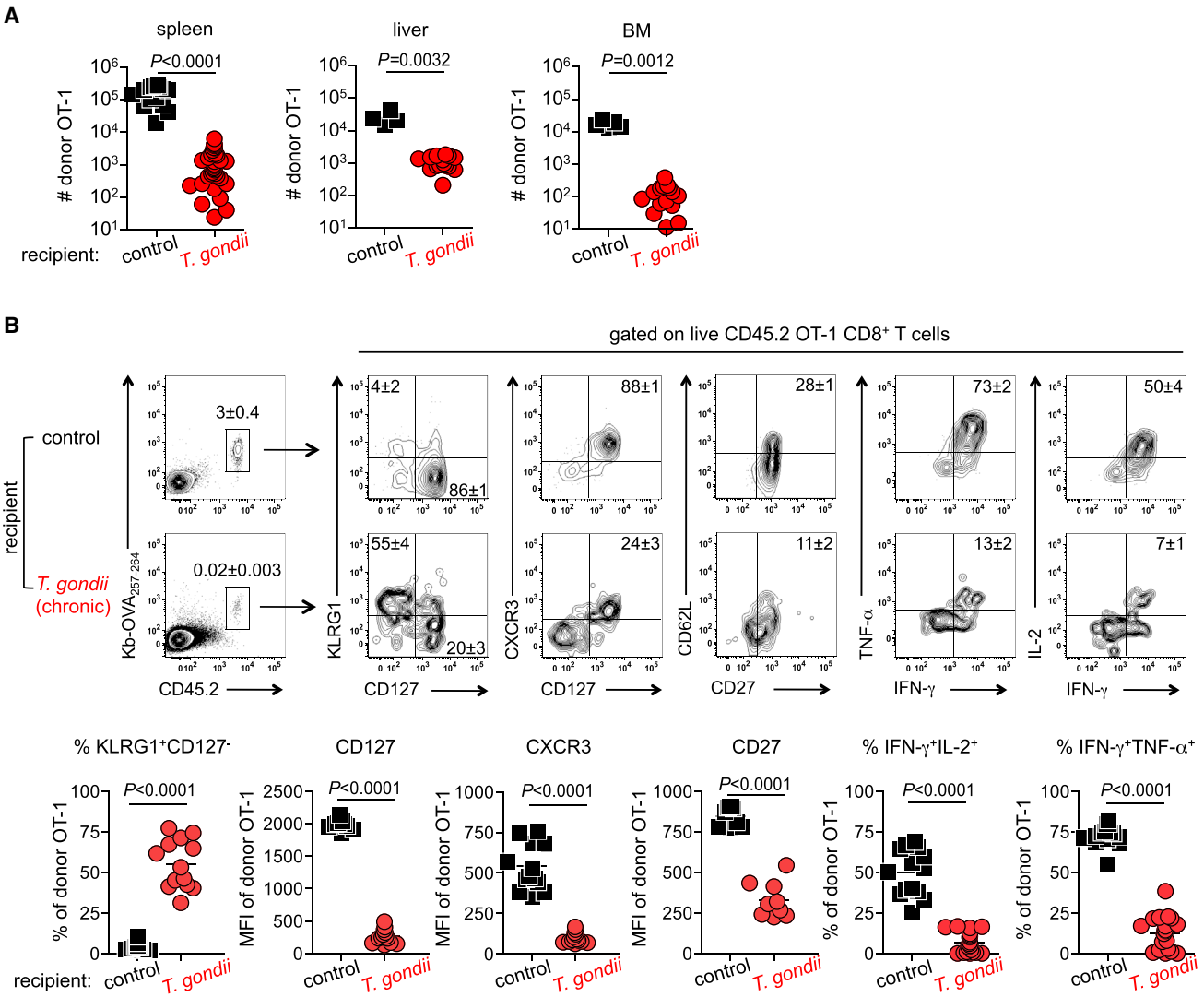

Figure 2. Bystander Chronic Parasitic Infection Disrupts the Effector to Memory Transition of CD8+ T Cells

CD45.2 OT1 CD8+ T cells were adoptively transferred to congenic CD45.1 mice. Recipients were infected with VSV-OVA. On d8 p.i., splenic CD45.2 OT1 CD8+ T cells were isolated and adoptively transferred to "control" (naive) or "*T. gondii*" (d8 post-*T. gondii* infection) recipient CD45.1 mice.

(A) OT1 cells were quantified in spleens, livers, and BM 30 days posttransfer.

(B) Phenotype and cytokine production of OT1 cells were analyzed 30 days posttransfer in spleens (n > 9). Similar results were obtained from blood, liver, and BM (data not shown). Numbers on FACS plots indicate frequencies of respective populations ± SEM. Statistical significance was calculated with two-tailed unpaired t test for normally distributed samples or Mann Whitney test for nonequally distributed samples.

Bystander Infections Impair Secondary Expansion and Protective Immunity of Memory CD8+ T Cells

Changes in the phenotypic markers of memory CD8+ T cell differentiation described above can be symptomatic of broader changes in the memory CD8+ T cell differentiation program. To test this, we examined two fundamental properties of memory CD8+ T cells: rapid recall response and protective immunity. Equal numbers of memory OT1 cells that had been exposed or not to bystander chronic *T. gondii* infection during their effector to memory differentiation (d8 until d40), were adoptively transferred to naive recipient mice that were subsequently challenged with LM-OVA. Upon secondary challenge, expansion of OT1 cells exposed to chronic infection was reduced ~10-fold compared to control OT1 cells (Figure 3A). When recipients were challenged with a higher dose of LM-OVA, control OT1 cells tended to provide better protection than OT1 cells exposed to bystander chronic infection, by increasing the median survival of recipient mice by 4.5 days (from 5.5 to 10 days) (Figure 3B). These results suggested that, in addition to causing attrition of memory CD8+ T cell numbers and skewing memory differentiation, bystander chronic infection impacted the secondary expansion and protective capacity of developing memory CD8+ T cells.

Chronic Bystander Infection Can Affect Preformed Memory CD8+ T Cells

To investigate whether these effects of bystander persisting infections were dependent on the differentiation stage of CD8+ T cells, we asked whether preformed memory CD8+ T cells

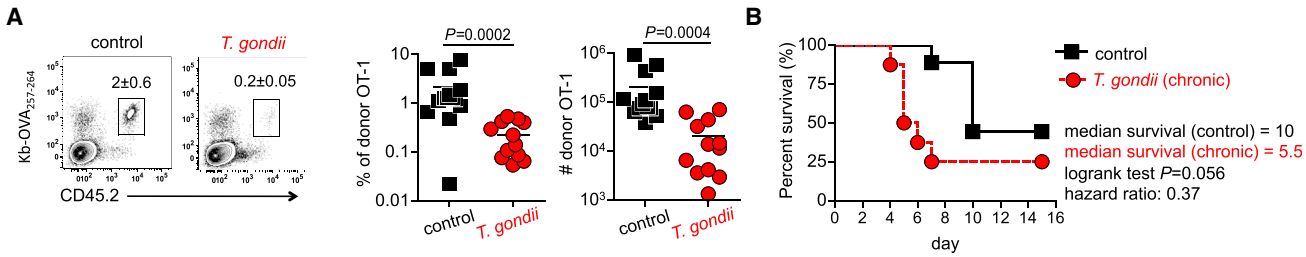

Figure 3. Bystander Chronic Infection Negatively Impacts Secondary Expansion and Protective Immunity of Memory CD8+ T Cells

CD45.2 OT1 CD8+ T cells were adoptively transferred to congenic CD45.1 mice. Recipients were infected with VSV-OVA. On d8 p.i., splenic CD45.2 OT1 CD8+ T cells were isolated and adoptively transferred to "control" (naive) or "*T. gondii*" (d8 post-*T. gondii* infection) recipient CD45.1 mice. Thirty days later, CD8+ T cells were purified and equal numbers (2×10^3) of donor OT1 cells were transferred to naive recipients that were subsequently challenged with LM-OVA. (To ensure equal potential transfer of inflammatory cells and/or mediators from *T. gondii*-infected mice, we mixed "control" cells with splenocytes from *T. gondii*-infected mice that did not contain OT1 cells and "chronic *T. gondii*" cells were mixed with splenocytes from naive mice that did not contain OT1 cells, at 1:1 ratio.)

(A) Expansion of donor OT1 cells was quantified in spleens 5 days after challenge with 8×10^4 LM-OVA (n > 12). Statistical significance was calculated with Mann-Whitney test.

(B) Survival of recipients that were challenged with 8×10^5 LM-OVA (n = 8–9).

would be impacted by bystander chronic infection. Thus, we adoptively transferred effector (d8 p.i.) or fully-formed memory (1 year p.i.) OT1 CD8+ T cells into naive or mice infected with LCMV clone 13 (Figure S5A). Persistence of memory CD8+ T cells was affected in chronically infected recipients (Figure S5B), in agreement with previous reports on the role of LCMV-induced attrition of preformed memory cells (McNally et al., 2001; Kim and Welsh, 2004). However, the effect of bystander chronic infection on the differentiation of preformed memory CD8+ T cells was not as prominent as the effect on transitioning d8 effector CD8+ T cells. For the effector CD8+ T cells transitioning to memory during bystander chronic infection, there was a profound reduction in both amount of CD127 and CXCR3 expressed at the cell surface, as well as the frequency of CD127+KLRG1− cells, as observed above. In contrast, although there was a decrease in the amount of CD127 and CXCR3 expressed per cell for fully-formed memory OT1 cells, the frequency of CD127+KLRG1− memory cells was not affected in the presence of bystander chronic infection (Figure S5C). These results suggest that fully-formed memory CD8+ T cells are less susceptible, though not impervious, to the effects of bystander chronic inflammation. These observations placed emphasis on understanding the particular potent impact of bystander chronic infections specifically during the critical phase of transitioning from effector to memory.

Chronic Bystander Infection Directly Impacts Memory Precursor CD8+ T Cells

Effector CD8+ T cell populations consist of memory precursors and terminally differentiated effector cells (Joshi et al., 2007). The effect of bystander chronic infection on the effector to memory transition could either be due to enhanced survival of terminal effectors or a direct impact on the developmental program of memory precursors. To distinguish between these possibilities, we purified memory precursor (CD127+KLRG1−) OT1 CD8+ T cells at d8 p.i. and adoptively transferred these cells into naive or recipients infected with the persistent strain LCMV clone 13 (Figure 4A). Recovery of OT1 memory CD8+ T cells 30 days later was reduced in mice infected with LCMV clone 13 (Figure 4B). Further, these cells had decreased expression of CD127 and moderately increased KLRG1 expression (Figure 4C). These results indicate a role for bystander chronic infection in directly impacting memory precursor CD8+ T cells in the posteffector phase by decreasing their survival and diverting their differentiation away from long-lived memory CD8+ T cells.

Bystander Infection Affects the Survival of Transitioning Memory CD8+ T Cells during Contraction

To interrogate the mechanism for the reduced recovery of transitioning OT1 memory CD8+ T cells in the presence of bystander persisting infection, we examined the survival of OT1 cells after in vitro culture with or without the T cell survival factor interleukin-7 (IL-7). OT1 cells that had been exposed to bystander chronic LCMV infection, survived less well in vitro with or without IL-7 (Figure S6A). We then examined pathways associated with contraction of effector CD8+ T cells to examine how these might be affected by bystander chronic infection. Contraction of CD127Lo terminal effector CD8+ T cells is mediated by the Bcl-2-interacting mediator of cell death (Bim) (Wojciechowski et al., 2006). We observed substantially increased expression of Bim in terminally differentiated KLRG1+CD127− OT1 cells that had been exposed to bystander chronic infection (Figure S6B), suggesting an increased propensity to die. On the other hand, central memory T cells have been shown to be more sensitive to TNF-α induced apoptosis compared to effector memory cells (Gupta et al., 2006) and TNF signaling through type 2 tumor necrosis factor receptor (TNFR2) was shown to also contribute to the contraction of CD8+ T cells upon influenza infection (Wortzman et al., 2013). We observed a significant increase in TNFR2 expression, specifically in the memory precursor subset (CD127+KLRG1−) of OT1 cells during bystander chronic infection (Figure S6B). Consistent with the results that bystander chronic infection skews the differentiation of memory precursor cells into terminally differentiated cells (see Figure 4), these results suggest a dual role of bystander chronic infection in affecting survival during the transition of effector CD8+ T cells to memory; CD8+ T cells are skewed to a more terminal effector phenotype and survival of both memory precursors and terminally differentiated cells is decreased in the presence of bystander chronic infection.

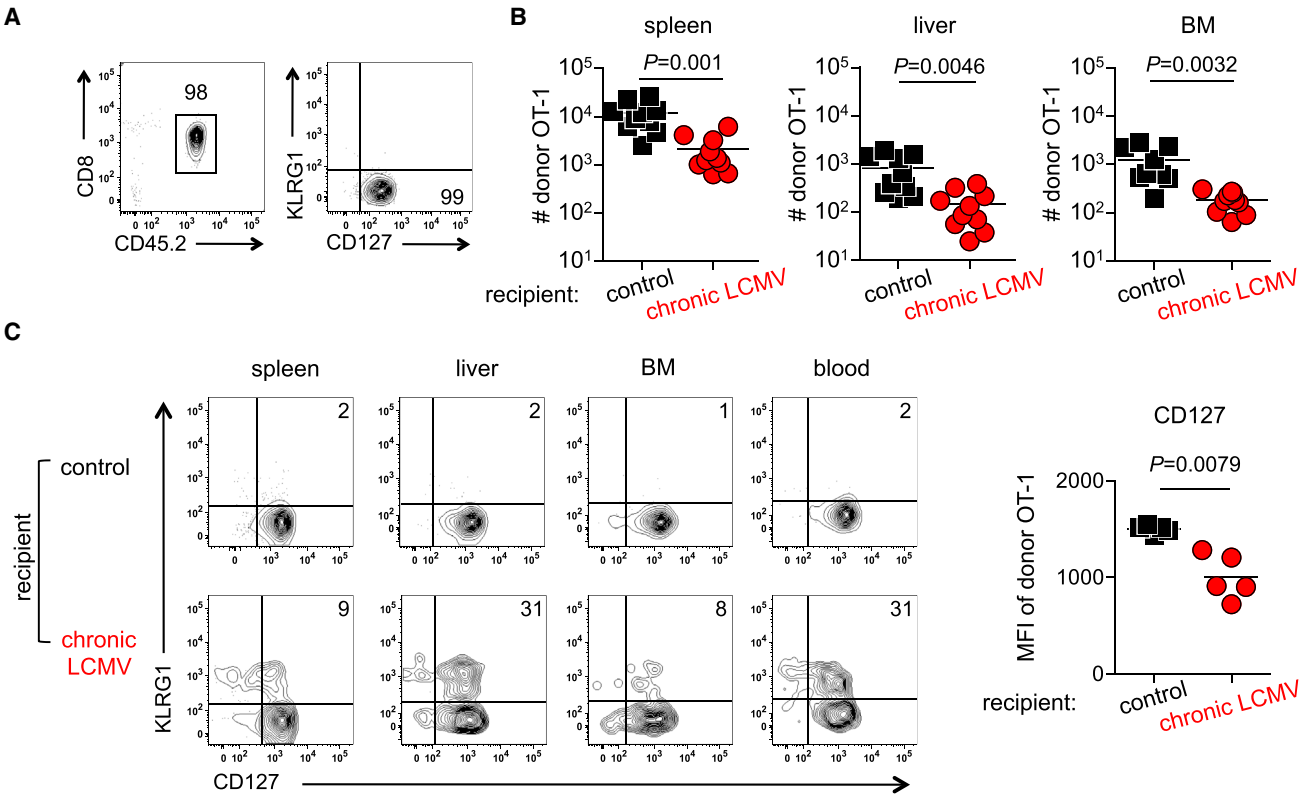

Figure 4. Bystander Chronic Infection Affects Memory Precursor CD8+ T Cells

CD45.1 mice containing CD45.2 OT1 cells were infected with VSV-OVA. On d8 p.i., CD127+KLRG1− CD45.2 OT1 CD8+ T cells were purified by FACS and adoptively transferred to "control" (naive) or "chronic LCMV" (d8 post-LCMV clone 13 infection) recipient CD45.1 mice.

(A) Purity of sorted OT1 cells.

(B) Numbers of OT1 cells were determined on d30 posttransfer (n > 9). Statistical significance was calculated with two-tailed unpaired t test.

(C) Phenotype of OT1 cells on d30 posttransfer (n > 9). FACS plots are gated on live OT1 CD45.2 CD8+ T cells and numbers indicate frequencies of respective populations. Statistical significance was calculated with two-tailed Mann Whitney test.

A Prominent Signature of Inflammatory Pathways in Memory CD8+ T Cells during Bystander Infection

To investigate the molecular mechanisms underlying the impact of bystander infection in skewing memory CD8+ T cell differentiation, we analyzed the gene-expression profiles of OT1 memory cells that had been exposed to bystander chronic LCMV infection during their transition from effector to memory. The transcriptional signature of purified OT1 memory cells exposed to chronic bystander infection was distinct from memory CD8+ T cells that developed in the control mice and revealed prominent upregulation of IFN-I-responsive genes (Figure 5A). By using Gene Set Enrichment Analysis (GSEA; Subramanian et al., 2005) we asked whether previously defined transcriptional signatures of inflammatory pathways or infections showed enrichment toward the transcriptional profile of OT1 cells exposed to bystander chronic infection (from Figure 5A). First, we compared these OT1 transcriptional signatures to previously defined transcriptional signatures of IFN-I and IL-12-regulated genes (Agarwal et al., 2009). The transcriptional signatures of T cells stimulated in vitro with IFN-I or IL-12 showed a robust enrichment toward the OT1 cells exposed to bystander chronic LCMV infection (Figure 5B). Further, inflammatory transcriptional signatures associated with TLR stimulation, such as those induced in dendritic cells by lipopolysaccharide (LPS) or R848 stimulation (Napolitani et al., 2005) and by CpG or Pam3CSK4 stimulation (Amit et al., 2009) also showed a significant enrichment in the transcriptional profile of OT1 cells exposed to bystander chronic LCMV infection (Figures 5C and 5D). Thus, transcriptional profiling of memory CD8+ T cells exposed to bystander persisting LCMV infection during the effector to memory transition suggested a prominent imprint of prolonged microbial stimulation and/or exposure to inflammatory cytokines.

These global gene-expression profiles also allowed us to ask whether the effects of bystander chronic infection extended beyond mouse models. The transcriptional signatures of human peripheral blood mononuclear cells (PBMC) from several viral and microbial infections were significantly enriched in the OT1 cells exposed to chronic LCMV infection (Figure 5E), suggesting that transcriptional changes associated with the bystander effect of CD8+ T cell memory in mice could be found in multiple infections in humans. These results also suggested that although acute inflammation is beneficial during priming, that same inflammatory signature might become detrimental if it is prolonged beyond the priming phase. We extended these human studies by generating the transcriptional signatures of cytomegalovirus (CMV)-specific CD8+ T cells from individuals with

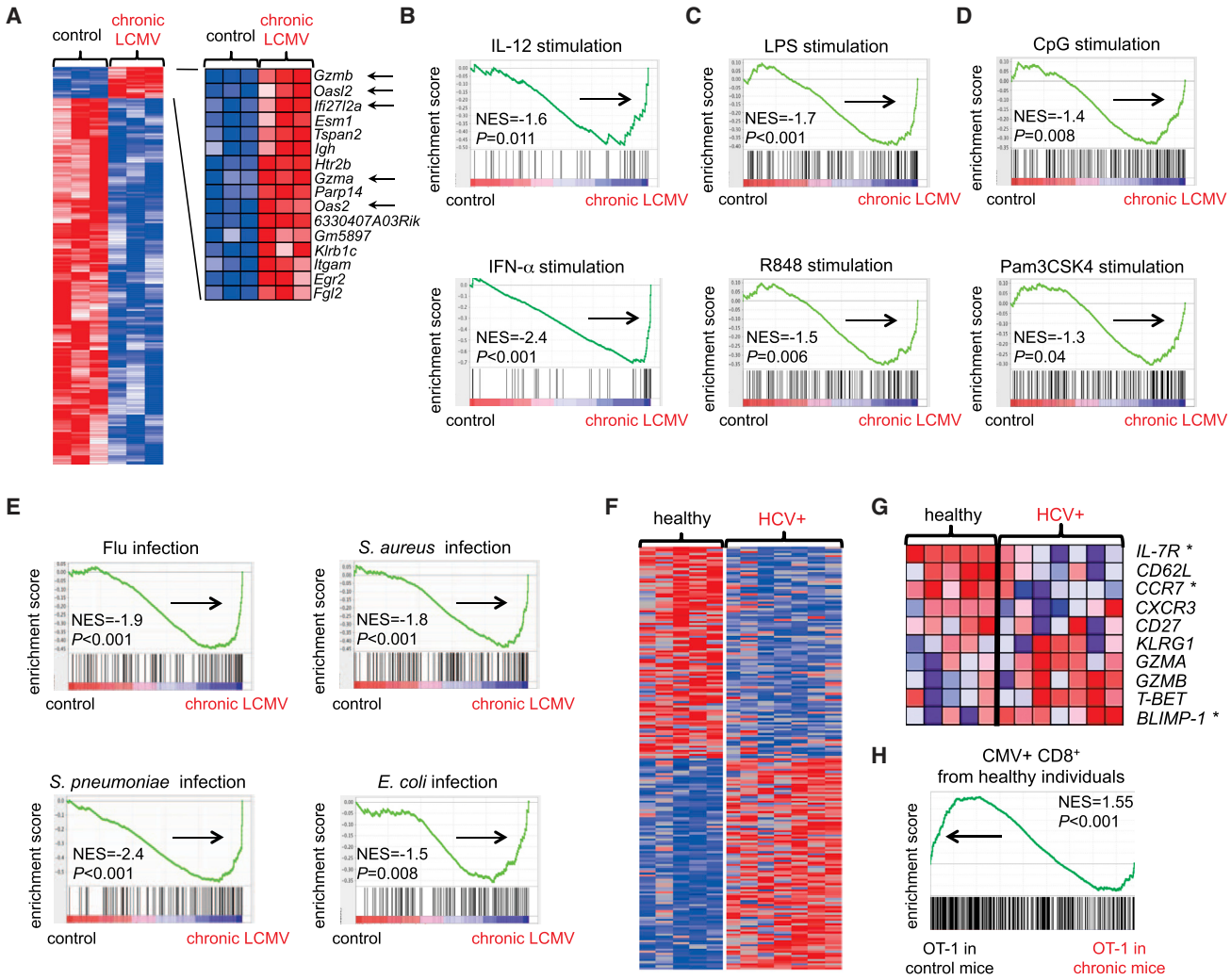

Figure 5. Gene-Expression Profiling Reveals a Prominent Imprint of Inflammatory Pathways in Memory Cells Exposed to Bystander Chronic Infection

(A) CD45.2 OT1 CD8+ T cells were primed in congenic CD45.1 mice following infection with VSV-OVA. On d8 p.i., splenic CD45.2 OT1 CD8+ T cells were isolated and adoptively transferred to "control" (naive) or "chronic LCMV" (d8 post-LCMV-infection) recipient CD45.1 mice. After 30 days, CD45.2 OT1 cells were sorted and microarray analysis was performed (n = 3). Heat map shows differentially expressed genes (>1.2-fold change, $p < 0.05$) in OT1 cells from "control" versus "chronic LCMV" mice. Arrows indicate IFN-I-regulated genes.

(B–E) GSEA was performed to compare: (B) the sets of upregulated genes in T cells during differentiation in vitro in the presence of IL-12 or IFN-I or 48 hr (Agarwal et al., 2009), (C) the sets of upregulated genes in dendritic cells upon stimulation with LPS or R848 for 2 hr (Napolitani et al., 2005), (D) the sets of upregulated genes in dendritic cells upon stimulation with CpG or Pam3CSK4 for 1 hr (Amit et al., 2009), (E) the sets of upregulated genes in human PBMC upon infection (Ramilo et al., 2007) with the transcriptional profile of memory OT1 CD8+ T cells purified from "control" or "chronic LCMV" mice (from Figure 5A). Normalized enrichment scores (NES) with statistical significance (p values), were calculated by GSEA. A negative enrichment score suggests that the transcriptional signature of interest was enriched toward the transcriptional profile of OT1 cells exposed to bystander chronic LCMV infection.

(F–H) Human CMV-specific CD8+ T cells were purified from PBMC obtained from subjects with persistent HCV viremia (infected for >1 year) and from healthy volunteers. (F) Heatmap shows top 50 genes differentially expressed between CMV-specific CD8+ T cells from healthy versus HCV-infected subjects. (G) Gene expression of memory-associated genes in CMV-specific CD8+ T cells from healthy and HCV-infected individuals. *$p < 0.05$. (H) Enrichment plot by GSEA comparing the set of upregulated genes (>2-fold) in CMV-specific CD8+ T cells from healthy individuals with the transcriptional profile of memory OT1 cells purified from "control" or "chronic LCMV" mice (from Figure 5A).

bystander chronic HCV infection and healthy controls (Figure 5F). Human CMV-specific (HLA-A*0201-NLVPMVATV or HLA-B*0702-TPRVTGGGAM tetramer-positive) CD8+ T cells were purified from PBMC of subjects with persistent HCV viremia (infected for >1 year) or from healthy volunteers and transcriptional profiling performed (Table S1). These gene-expression profiles revealed a clear impact of bystander chronic HCV infection on CMV-specific CD8+ T cells, including differential expression of many key CD8+ T cell memory-related genes (Figure 5G). The transcriptional signature of CMV-specific CD8+ T cells from healthy individuals was enriched in the OT1 CD8+ T cells from control, but not from chronically infected mice (Figure 5H),

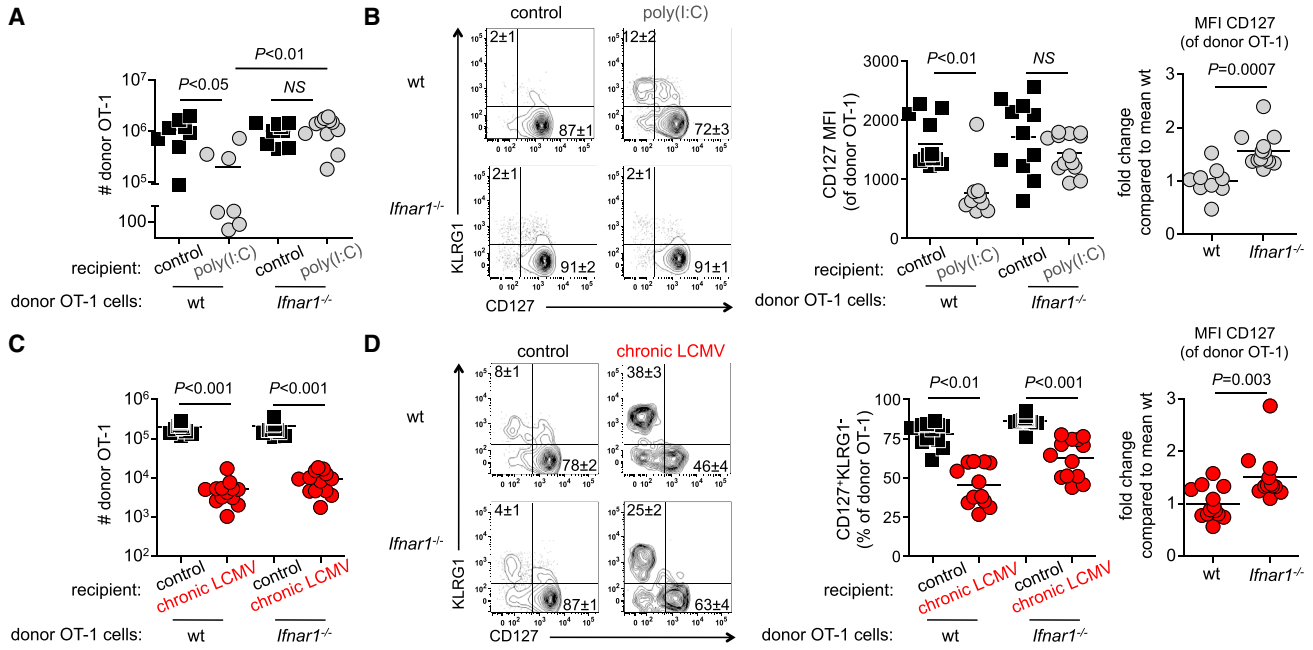

Figure 6. Prolonged IFNAR Signaling Disrupts the Transition of CD8+ T Cells from Effector to Memory

(A and B) WT or *Ifnar1*$^{-/-}$ CD45.2 OT1 CD8+ T cells were primed in recipient CD45.1 mice upon infection with VSV-OVA. Starting on d8 p.i., mice were administered 100 μg poly(I:C) or PBS (control) i.p. every 3 days. (A) Number of OT1 cells was determined in spleens on d40 p.i. (n > 10). Similar results were obtained from livers and BM (data not shown). Statistical significance was calculated with two-tailed Mann Whitney test. (B) Phenotype of OT1 cells was analyzed in spleens on d40 p.i. (n > 10). Similar results were obtained from livers and BM (data not shown). MFI of CD127 on donor OT1 cells in poly(I:C)-treated mice was normalized to the mean measurements of WT OT1 cells in poly(I:C)-treated mice from each experiment. Statistical significance was calculated with two-tailed unpaired t test.

(C and D) CD45.1 mice containing adoptively transferred CD45.2 *Ifnar1*$^{-/-}$ (n = 12) or littermate control WT OT1 CD8+ T cells were infected with VSV-OVA. On d8 p.i., OT1 cells were adoptively transferred to "control" (naive) or "chronic LCMV" (at d8 post-LCMV clone 13 infection) recipient mice. (C) Numbers and (D) phenotype of donor OT1 cells were analyzed in spleens 40 days later. MFI of CD127 on donor OT1 cells in "chronic LCMV" mice was normalized to the mean measurements of WT OT1 cells in "chronic LCMV" mice from each experiment. FACS plots are gated on live OT1 CD45.2 CD8+ T cells and numbers indicate frequencies ± SEM. n.s., not significant.

consistent with the findings that chronic viral and parasitic infections in mouse models skew bystander CD8+ T cells away from normal memory development. These studies revealed a fundamental shared phenomenon of many bystander chronic infections that transcends species and infection type. Moreover, these data implicate chronic exposure to inflammation and downstream inflammation-driven gene expression.

IFN-I Impairs the Transition from Effector to Memory in the Absence of Infection

The robust enrichment of IFN-related genes in CD8+ T cells undergoing memory differentiation in the presence of chronic bystander infections led us to examine the impact of prolonged induction of IFN-I and related inflammatory pathways on memory CD8+ T cell development in the absence of chronic infection. Serial infections can cause attrition of preformed memory CD8+ T cells, an effect attributed to IFN-I (McNally et al., 2001; Kim and Welsh, 2004). However, the impact of chronic IFN-I on effector CD8+ T cells transitioning to memory, as well as the effects on the differentiation program of the resulting memory CD8+ T cells remain poorly understood. To examine this issue, we infected mice containing OT1 cells with VSV-OVA. Starting on d8 p.i., mice were treated every 3 days with poly(I:C), a potent inducer of IFN-I and other inflammatory pathways. Prolonged exposure to poly(I:C) resulted in a progressive loss of OT1 cells (Figure 6A), consistent with the previously described IFN-I-induced attrition (McNally et al., 2001; Kim and Welsh, 2004). However, this treatment also caused a skewed pattern of terminal effector CD8+ T cell differentiation (KLRG1+CD127−) (Figure 6B). These effects were dependent on the expression of IFNAR on CD8+ T cells, because *Ifnar1*$^{-/-}$ OT1 CD8+ T cells developed into CD127+KLRG1− memory cells, even in the presence of poly(I:C)-induced chronic inflammation (Figures 6A and 6B). Similar results were observed following priming with LM-OVA, instead of VSV-OVA (data not shown). Thus, prolonged exposure to poly(I:C)-induced inflammation after clearance of the primary infection recapitulated key aspects of bystander chronic infection, suggesting that chronic inflammatory signals can skew memory development, even in the absence of pathogen.

IFNAR Signaling in CD8+ T Cells Is Crucial during poly(I:C) Treatment but Not Bystander Infection

Although IFN-I is rapidly induced early during many infections, IFN-I production rapidly declines during some chronic infections including LCMV (Zuniga et al., 2008; Ye and Maniatis, 2011), raising the question of whether IFN-I alone could be the signal that caused altered CD8+ T cell memory development during chronic bystander infections. To directly test this possibility,

we examined whether signals through IFNAR were required for the skewed memory CD8+ T cell differentiation during bystander chronic LCMV infection. We adoptively transferred *Ifnar1*$^{-/-}$ OT1 effector CD8+ T cells into control mice or mice infected with persistent LCMV. Absence of IFNAR on OT1 cells did not restore the numerical defects induced by bystander infection (Figure 6C) and led to only a minor impact on the differentiation of OT1 cells during chronic LCMV infection (Figure 6D), despite fully reversing the effect of chronic poly(I:C) administration (Figures 6A and 6B). These results suggested that CD8+ T cell intrinsic IFN-I signaling alone could not fully explain the impact of bystander chronic LCMV infection on memory CD8+ T cell differentiation. Lack of IFN-γR or IL-12R on OT1 cells also did not restore normal memory CD8+ T cell numbers or differentiation during bystander chronic LCMV infection (data not shown). Additionally, by using IFN-γ blocking antibodies in vivo in combination with *Ifnar1*$^{-/-}$ OT1 cells did not improve OT1 memory development (data not shown). Because IFN-α, IFN-β, IFN-γ, and IL-12 can have redundant effects during priming of CD8+ T cells (Haring et al., 2006; Curtsinger and Mescher, 2010), it was possible that different inflammatory pathways had overlapping roles in the detrimental effects of bystander chronic infection. This notion is also consistent with the effects of bystander chronic infection on memory CD8+ T cell development observed during different chronic infections, including the IFN-inducing chronic LCMV, the IL-12-inducing *T. gondii*, and the Th2-type *H. polygyrus* infections.

T-bet and Blimp-1 Regulate Defective Transitioning to Memory during Bystander Chronic Infection

Diverse inflammatory signals can influence T cell differentiation by accessing common transcriptional coordinators of differentiation. Two such transcription factors, T-bet and Blimp-1, are known to have a role in integrating inflammation and antigen signals during priming (reviewed in Kaech and Cui, 2012). However, it is not clear whether these transcription factors have a role in the antigen-independent phase of memory CD8+ T cell differentiation. To test this idea, we used *Prdm1*$^{fl/fl}$ *Gzmb*-Cre mice (in which Blimp-1 was deleted only in CD8+ T cells) or T-bet-heterozygous (*Tbet*$^{+/-}$) effector OT1 CD8+ T cells, generated with VSV-OVA priming. We then adoptively transferred these effector cells into control or mice infected with persistent LCMV and examined the effector to memory transition. The total number of either Blimp-1 or T-bet deficient OT1 CD8+ T cells remained reduced in the mice infected with LCMV clone 13 compared to controls (Figure S7), suggesting that the Bim and TNFR2 effects on the contraction of developing bystander memory CD8+ T cells are independent of these transcription factors. However, Blimp-1 or T-bet deficiency completely abrogated the negative impact of bystander infection on memory CD8+ T cell differentiation, because *Prdm1*$^{fl/fl}$ *Gzmb*-Cre and *Tbet*$^{+/-}$ OT1 cells differentiated into CD127+KLRG1−CXCR3+ memory CD8+ T cells in the presence of bystander infection (Figure 7). Thus, Blimp-1 and T-bet, in addition to transiently integrating antigen and inflammation during the effector T cell expansion (Kaech and Cui, 2012), are also critically involved in sensing prolonged inflammation in the absence of antigen and skewing memory CD8+ T cell differentiation during bystander chronic infections. Together, these data suggest a model where bystander chronic infection and prolonged inflammation impact long-term survival, as well as differentiation of developing memory CD8+ T cells through independent mechanisms.

DISCUSSION

We revealed a pervasive impact of bystander chronic infections and inflammation on immunological memory in multiple settings. Specifically, we found a major unappreciated effect on the transition of optimally primed effector CD8+ T cells into robust memory CD8+ T cell populations. Distinct effects of bystander chronic infection and inflammation on the number of memory CD8+ T cells and on the differentiation program of long-lived memory CD8+ T cells were revealed. The latter effect could be restored by specific CD8+ T cell-intrinsic deficiency of T-bet or Blimp-1. Chronic poly(I:C) treatment recapitulated the known role of IFN-I-associated attrition in viral infections, and a prominent signature of IFN-I-inducible genes pointed to an effect of IFN-I. However, although intrinsic IFNAR deficiency in CD8+ T cells abrogated the effect of chronic poly(I:C) treatment, it did not reverse the effect of bystander chronic LCMV infection. These observations collectively suggest that during actual chronic bystander infection, multiple inflammatory pathways might alter the ability of effector CD8+ T cells to efficiently transition to memory and develop canonical memory CD8+ T cell properties. Our mouse studies are supported by human CMV responses. Although it is not possible to define the timing of CMV versus HCV acquisition or exclude potential changes in the reactivation or viral activity of CMV, the persisting "smoldering" nature of CMV infection suggests that at any given time, some fraction of the CMV-specific CD8+ T cells will be in transition from recent antigen encounter and an "effector" status to resting memory, allowing the imprint of HCV infection on these differentiation events to be detected. It is possible that there might be more CMV replication in the setting of chronic HCV infection, a possibility that might warrant further investigation, but the impact on memory CD8+ T cell differentiation was similar to that observed in the mouse models, suggesting a bystander effect of chronic HCV. It should be noted that while we observed an increase in transcripts encoding memory-associated genes expressed by CMV-specific CD8+ T cells in healthy versus HCV-infected subjects, this does not mean that these cells are more "central memory"-like, because this is only a relative comparison and the CMV-specific CD8 T cells from healthy controls expressed lower amounts of these transcripts compared to naïve T cells. Thus, although the precise pathogenesis of CMV and HCV is different than the mouse pathogens used here, the imprint of bystander chronic infection and prolonged inflammation is conserved between species. Although our studies support a negative impact of different Th1 and Th2-inducing viral and parasitic bystander chronic infections on memory CD8+ T cell development, the effect of other types of infections and/or chronic inflammatory conditions remains to be investigated. Coinfections have long been thought to influence innate immunity, naive to effector T cell differentiation, and skewing of helper T cell lineage (e.g., Th1 versus Th2). These current observations place major emphasis on memory formation and the effector to memory transition as a critical effect of coinfection on long-term immune memory.

Initial priming of CD8+ T cells requires three signals: signal 1 from antigen, signal 2 from costimulation, and signal 3 from

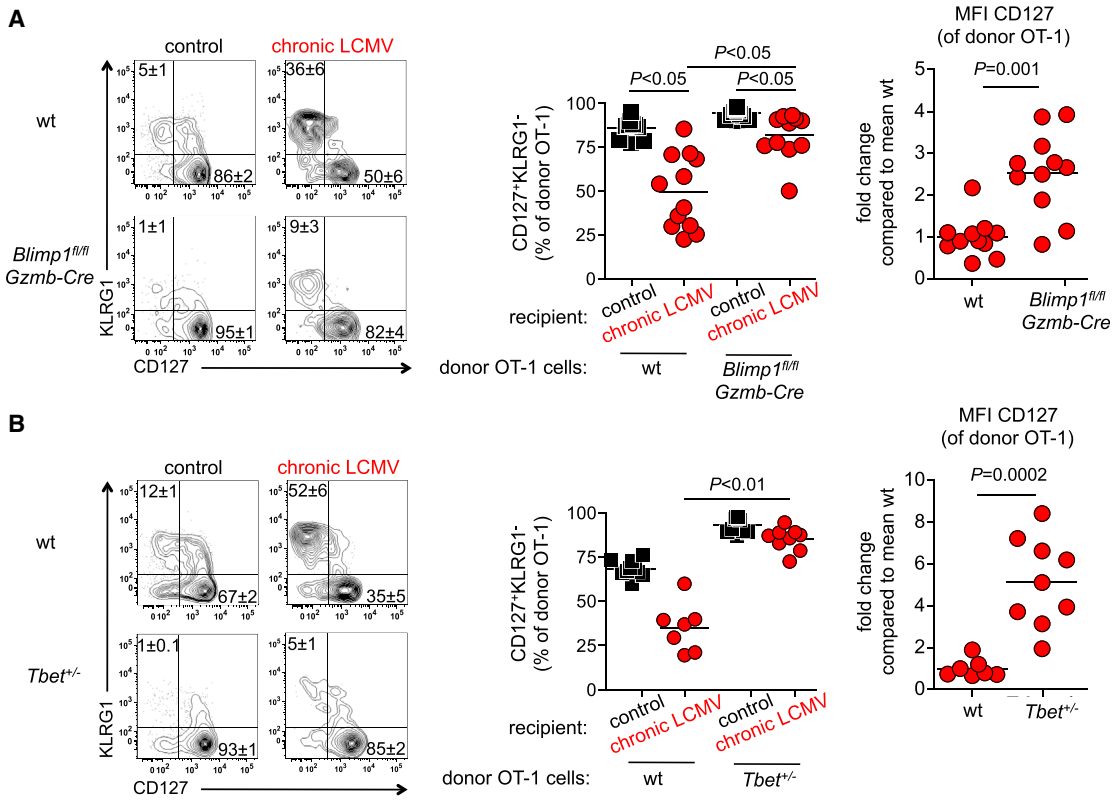

Figure 7. T-bet and Blimp-1 Regulate Memory CD8+ T Cell Development during Chronic Bystander Infection

CD45.1 mice containing adoptively transferred CD45.2 *Prdm1*$^{fl/fl}$ *Gzmb*-Cre (n = 11) (A), *Tbet*$^{+/-}$ (n = 9) (B), or littermate control WT OT1 CD8+ T cells were infected with VSV-OVA. On d8 p.i., OT1 cells were adoptively transferred to "control" (naive) or "chronic LCMV" (at d8 post-LCMV clone 13 infection) recipient mice. Donor OT1 cells were analyzed in spleens 40 days later. FACS plots are gated on live OT1 CD45.2 CD8+ T cells and numbers indicate frequencies ± SEM. MFI of CD127 on donor OT1 cells in "chronic LCMV" mice was normalized to the mean measurements of WT OT1 cells in "chronic LCMV" mice from each experiment.

inflammatory cytokines such as IFN-α and −β, IL-12, IL-1, and IL-33 (Haring et al., 2006; Curtsinger and Mescher 2010; Bonilla et al., 2012). Previous studies have demonstrated that developing effector CD8+ T cells integrate signals from antigen, costimulation, and inflammation to guide the development and differentiation of effector T cells (reviewed in Haring et al., 2006; Curtsinger and Mescher 2010). However, the positive effects of inflammatory signals during priming on the expansion of effector CD8+ T cells can occur at the expense of the quantity and quality of the generated memory CD8+ T cells (Badovinac et al., 2005; Joshi et al., 2007; Pearce and Shen, 2007). Our results further indicate that the effects of inflammation can be uncoupled from antigen stimulation and demonstrate that developing memory CD8+ T cells continue to sense inflammation after antigen clearance. Specifically, this scenario of prolonged antigen-independent sensing of high amounts of inflammation affects the transition of effector CD8+ T cells into robust long-lived memory CD8+ T cells by skewing the pattern of differentiation.

Although there is some evidence from in vitro models that sensing of inflammation changes once TCR signaling has ceased (Schulz et al., 2009), the mechanisms by which inflammation regulates CD8+ T cell memory without concomitant antigen signals in vivo remain poorly understood. Here, we show that the transcription factors T-bet and Blimp-1 integrate inflammatory signals and disrupt the efficient transitioning of effector CD8+ T cells into the memory pool in the absence of ongoing antigen stimulation. Although genetic deletion of Blimp-1 or T-bet led to a dramatic recovery of memory CD8+ T cell differentiation, a numerical defect persisted, suggesting at least two independent effects of bystander chronic infection on establishment of memory CD8+ T cell numbers and differentiation. However, the restoration of memory CD8+ T cell differentiation when Blimp-1 or T-bet were absent or reduced suggests that targeting these transcriptional pathways could potentially improve memory CD8+ T cell development during bystander chronic infection.

Inflammatory signals such as IFN-α, IFN-β, and IFN-γ are crucial in the first few days of infection to initiate a robust antiviral effector CD8+ T cell response (Haring et al., 2006; Curtsinger and Mescher 2010). However, transient IFN-I produced during acute viral infections can inhibit antigen-driven proliferation of preformed bystander memory CD8+ T cells (Marshall et al., 2011). IFN-α, IFN-β, and IFN-γ induced by serial infections has also been implicated in attrition of preformed memory cells in some settings (McNally et al., 2001; Dudani et al., 2008), although attrition of preformed memory CD8+ T cells following repeated acute infections or vaccinations is not always observed (Vezys et al., 2009; Odumade et al., 2012). Thus, the role of IFN pathways in regulating CD8+ T cell memory remains controversial. Despite the persistence of an IFN-I inducible gene signature during chronic infections, sustained IFN-I production is impaired

(Zuniga et al., 2008; Ye and Maniatis, 2011) raising questions about what inflammatory pathways govern this bystander effect during chronic infections. Indeed, removal of IFNAR on CD8+ T cells had only moderate impact on reversing the negative effect of bystander chronic LCMV infection on memory CD8+ T cell development, suggesting involvement of additional pathways. Although other inflammatory pathways could not be individually implicated, preventing the ability to integrate different inflammatory signals via Blimp-1 or T-bet reversed the differentiation defects caused by bystander chronic infection. Thus, our data are consistent with a model where multiple inflammatory pathways act redundantly during chronic infections and cause skewing of the effector to memory transition.

These studies could have implications not only for understanding memory development but also for global health. Some persisting infections confer intrinsic resistance to unrelated infections (Barton et al., 2007; Vahidnia et al., 2012). However, the data presented here suggest that chronic coinfections and inflammation might be a substantial impediment to achieving optimal immunological memory. While IFN-I is clearly antiviral during the early stages of infection, several studies have found a negative association between prolonged IFN-I signaling and the outcome of chronic infections (Antonelli et al., 2010; Berry et al., 2010; Rotger et al., 2011; Fraietta et al., 2013). Moreover, blockade of IFNAR during chronic viral infection has recently been found to paradoxically benefit antiviral adaptive immune responses (Teijaro et al., 2013; Wilson et al., 2013). Several mechanisms might be involved in these settings and it is unclear whether signaling through IFNAR is always required for an IFN-I-inducible gene signature. For example, there is considerable overlap in the genes induced by IL-12 and IFN-I in some settings (Agarwal et al., 2009). However, our data suggest that this IFN-I signature is at least a symptom of the bystander effect and that impaired immunological memory might contribute to negative association of the IFN-I signature and outcome of some chronic infections. Therefore, these observations are relevant for vaccination of patients in developing countries or those with chronic inflammatory diseases, as well as for long-term IFN-I-based treatment of infectious disease. This study suggests opportunities to optimize these vaccination and therapeutic strategies by further understanding the impact of chronic coinfections and prolonged inflammation on immunological memory.

EXPERIMENTAL PROCEDURES

Mice

Four to eight-week-old C57BL/6 Ly5.2CR (CD45.1) mice were purchased from NCI and TCR transgenic OT1 C57BL/6 mice from Jackson Laboratories. IFNAR-deficient (*Ifnar1*$^{-/-}$), IL-12Rβ-deficient (*Il12rb*$^{-/-}$), *Tbx21*-heterozygous (*Tbet*$^{+/-}$), and *Prdm1*$^{flox/flox}$ (Blimp-1) crossed to *Gzmb*-Cre mice were bred with OT1 mice. *Ifngr*$^{-/-}$ CD8+ T cells were provided by Jason Whitmire (University of North Carolina). Animal procedures were performed in accordance with Institutional Animal Care and Use Committee guidelines for the Wistar Institute and University of Pennsylvania.

Viruses and Bacteria

Mice were infected intravenously (i.v.) with 4 × 10^6 PFU LCMV clone 13, intraperitoneally (i.p.) with 2 × 10^5 PFU LCMV Armstrong, i.p. with 250 *T. gondii* tachyzoites (Me49 B7 strain), i.v. with 2 × 10^6 VSV-OVA, or i.p. with 10^4 – 8 × 10^5 cfu LM-OVA, as indicated. LM-OVA was grown and used as described (Pearce and Shen, 2007).

Adoptive Transfer

For the generation of primary OT1 effector cells, 5 × 10^4 CD45.2 CD8+ OT1 cells were adoptively transferred i.v. to WT CD45.1 recipient mice followed by infection with 2 × 10^6 PFU VSV-OVA or with 10^4 cfu LM-OVA. On d8 p.i. splenic CD8+ T cells were purified by negative selection with magnetic beads (Miltenyi Biotec) according to the manufacturer's instructions and 10^7 effector OT1 cells were adoptively transferred i.v. to congenic CD45.1 recipient mice.

Flow Cytometry

Tissues were processed, single cell suspensions obtained, and cells were stained as described (Wherry et al., 2003). Cells were stained with LIVE/DEAD cell stain (Invitrogen), CD8 (Abcam), CD44, CD45.2, CD62L, and CD27 (Biolegend), CD127 (eBiosciences), KLRG1 (Cell Lab), CXCR3 (R&D Systems), TNFR2 (BD PharMingen), Bim (Cell Signaling), and MHC class I/K^b $OVA_{257–264}$ tetramer. Intracellular cytokine staining was performed after 5 hr of ex vivo stimulation with $OVA_{257–264}$ peptide as described (Wherry et al., 2003) and cells stained with IL-2, TNF-α (Biolegend), and IFN-γ (eBioscience). Cells were analyzed with LSRII (BD Biosciences) and FlowJo software (Treestar).

Gene-Expression Profile Analysis

CD8+ T cells were enriched with magnetic beads and CD45.2 OT1 CD8+ T cells were sorted on a FACSAria (BD Biosciences). RNA was isolated with TRIzol (Invitrogen) according to the manufacturer's instructions. RNA was processed, amplified, labeled, and hybridized to Affymetrix GeneChip MoGene 1.0 ST microarrays at the Molecular Profiling Facility of the University of Pennsylvania. Human CMV-specific (HLA-A*0201-NLVPMVATV or HLA-B*0702-TPRVTGGGAM tetramer-positive) CD8+ T cells were purified from PBMC obtained from subjects with persistent HCV viremia (infected for >1 year) or from healthy volunteers who were recruited at Massachusetts General Hospital in Boston (Table S1). The study was approved by the local IRB (Protocol # 1999-P-004983/54; MGH #: 90-7246). RNA was isolated, processed, amplified, labeled, and hybridized to Affymetrix Human Gene 1.0 ST microarrays. Affymetrix Power Tools software was used to process and quantile normalize fluorescent hybridization signals with the Robust Multichip Averaging method (Irizarry et al., 2003). Transcripts were $\log_2$ normalized. Hierarchical Clustering was performed with Gene Pattern (Reich et al., 2006), and Gene Set Enrichment Analysis was performed with GSEA software (Subramanian et al., 2005).

Statistical Analysis

Samples were tested for normal distribution using D'Agostino and Pearson normality test. For equally distributed samples, statistical significance was calculated with unpaired two-tailed t test or one-way ANOVA (with Bonferroni posttest), as indicated. For nonequally distributed samples or samples too small to test for normal distribution, nonparametric Mann-Whitney test or Kruskal-Wallis (with Dunn's posttest) test was performed, as indicated.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2014.04.010.

ACKNOWLEDGMENTS

We thank the Penn Molecular Profiling Facility at the University of Pennsylvania for conducting the mouse microarray experiment. We thank D. Herbert (UCSF) for the *H. polygyrus* and members of the Wherry laboratory for comments and input. This work was supported by NIH/NIAID grants AI 083022 (to H.S. and E.J.W.) and AI 082630 (to W.N.H., G.M.L., and E.J.W.).

Received: August 19, 2013
Accepted: March 11, 2014
Published: May 15, 2014

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Supplemental Information

Bystander Chronic Infection Negatively Impacts Development of CD8$^+$ T Cell Memory

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Supplemental Figure 1. Bystander chronic LCMV infection negatively impacts memory CD8$^+$ T cell development to unrelated antigens. (Related to Figure 1)

C57BL/6 mice were infected with LCMV clone 13 ("chronic LCMV") or left untreated ("control"). 30 days later, both groups were infected with 5 x 10^4 CFU LM-OVA. Memory CD8 T cell responses to OVA were measured in spleens using Kb-$OVA_{257-264}$ tetramer staining 30 days post-LM-OVA infection (n=10). Similar results were obtained from liver and BM. Numbers on FACS plots indicate frequencies of respective populations ± S.E.M. Normal distribution was tested with D'Agostino & Pearson normality test and statistical significance was calculated using two-tailed unpaired t-test for normally distributed samples and the non-parametric Mann Whitney test for non-equally distributed samples.

Supplemental Figure 2. Bystander chronic viral infection disrupts the effector to memory transition of CD8$^+$ T cells. (Related to Figure 1)

CD45.2 OT-1 CD8$^+$ T cells were adoptively transferred to congenic CD45.1 mice. Recipients were infected with 10^4 CFU LM-OVA. On d8 p.i., splenic CD45.2 OT-1 CD8$^+$ T cells were isolated and adoptively transferred to "control" (naïve) or "chronic LCMV" (d8 post-LCMV clone-13 infection) CD45.1 recipient mice. **(A)** OT-1 cells were quantified in spleens, livers and BM, 30 days post-transfer. **(B)** Phenotype and cytokine production by OT-1 cells were analyzed 30 days post-transfer in spleens (n>10). Similar results were obtained from blood, liver and BM (data not shown). **(C)** Equal numbers of CD45.1 splenocytes at d8 post-VSV-

OVA priming were adoptively transferred to CD45.2 “control” (naïve) or “chronic LCMV” (d8 post-LCMV clone-13 infection) recipient mice. Numbers of $CD8^+$ CD45.1 donor cells were calculated in the spleen, liver, inguinal lymph nodes (iLN) and BM at d2 post-transfer. Total number of CD45.1 $CD8^+$ donor cells was calculated as the sum of numbers of CD45.1 $CD8^+$ T cells detected in spleen, liver, iLN and BM. For BM, two femurs were analyzed and the number of CD45.1 $CD8^+$ cells was multiplied by 7.9 to account for total BM in the animal (as described by Slifka et al., 1995). Numbers on FACS plots indicate frequencies of respective populations ± S.E.M. Normal distribution was tested with D’Agostino & Pearson normality test and statistical significance was calculated using two-tailed unpaired t-test for normally distributed samples and the non-parametric Mann Whitney test for non-equally distributed samples.

Supplemental Figure 3. Established bystander chronic viral infection disrupts the effector to memory transition of $CD8^+$ T cells. (Related to Figure 1)

CD45.2 OT-1 $CD8^+$ T cells were adoptively transferred to congenic CD45.1 mice. Recipients were infected with VSV-OVA. On d8 p.i., splenic CD45.2 OT-1 $CD8^+$ T cells were isolated and adoptively transferred to “control” (naïve) or “chronic LCMV” (d30 post-LCMV clone-13 infection, **A-B**; d120 post-LCMV clone-13 infection, **C-D**) CD45.1 mice. **(A, C)** OT-1 cells were quantified in spleens, livers and BM, 30 days post-transfer. **(B, D)** Phenotype of OT-1 cells was analyzed 30 days post-transfer in spleens and livers (n>7). Similar results were obtained from

BM (data not shown). Numbers on FACS plots indicate frequencies of respective populations ± S.E.M. Normal distribution was tested with D'Agostino & Pearson normality test and statistical significance was calculated using two-tailed unpaired t-test for normally distributed samples and the non-parametric Mann Whitney test for non-equally distributed samples.

Supplemental Figure 4. Chronic *H. polygyrus* infection disrupts the effector to memory transition of CD8$^+$ T cells. (Related to Figure 2)

CD45.2 OT-1 CD8$^+$ T cells were adoptively transferred to congenic CD45.1 mice. Recipients were infected with VSV-OVA. On d8 p.i., splenic CD45.2 OT-1 CD8$^+$ T cells were isolated and adoptively transferred to "control" (naive) or "chronic *H. polygyrus*" (d15 post-*H. polygyrus* infection) CD45.1 mice. **(A)** OT-1 cells were quantified in spleen and BM 30 days post-transfer. **(B)** Phenotype and cytokine production by OT-1 cells were analyzed on d30 post-transfer in spleens (n>12). Similar results were obtained from blood, liver and BM (data not shown). Numbers on FACS plots indicate frequencies of respective populations ± S.E.M. Normal distribution was tested with D'Agostino & Pearson normality test and statistical significance was calculated using two-tailed unpaired t-test for normally distributed samples and the non-parametric Mann Whitney test for non-equally distributed samples.

Supplemental Figure 5. Chronic bystander infection affects pre-formed memory CD8$^+$ T cells. (Related to Figure 4)

CD45.2 OT-1 $CD8^+$ T cells were adoptively transferred to congenic CD45.1 mice. Recipients were infected with VSV-OVA. On d8 p.i. ("effector") or 1 year p.i. ("memory"), splenic CD45.2 OT-1 $CD8^+$ T cells were isolated and adoptively transferred to "control" (naive) or "chronic LCMV" (d8 post-LCMV clone 13 infection) CD45.1 mice. **(A)** Phenotype of OT-1 cells at the time of transfer. **(B)** OT-1 cells were quantified in spleen on d30 post-transfer. **(C)** Phenotype of OT-1 cells was analyzed on d30 post-transfer in spleens (n=5). Numbers on FACS plots indicate frequencies of respective populations.

Supplemental Figure 6. Bystander chronic viral infection affects the survival of $CD8^+$ T cells during the contraction phase. (Related to Figure 4)

CD45.2 OT-1 $CD8^+$ T cells were adoptively transferred to congenic CD45.1 mice. Recipients were infected with VSV-OVA. On d8 p.i., splenic CD45.2 OT-1 $CD8^+$ T cells were isolated and adoptively transferred to "control" (naïve) or "chronic LCMV" (d8 post-LCMV clone-13 infection) CD45.1 mice. 14 days post-transfer splenocytes were cultured *in vitro* with (right panel) or without (left panel) IL-7 (10ng/ml) for 48 h. (To ensure a comparable environment of inflammatory cells and/or mediators, "control" cells were mixed with splenocytes from LCMV-infected mice that did not contain OT-1 cells and "chronic LCMV" cells were mixed with splenocytes from naïve mice that did not contain OT-1 cells, at 1:1 ratio.) **(B)** Intracellular Bim and surface TNFR2 staining was performed *ex vivo* at d14 post-transfer. Numbers on histograms indicate percentage of Bim^+ cells of donor OT-1 ± S.E.M. Normal distribution was tested with D'Agostino & Pearson

normality test and statistical significance was calculated using two-tailed unpaired t-test for normally distributed samples and the non-parametric Mann Whitney test for non-equally distributed samples.

Supplemental Figure 7. Blimp-1 or T-bet deficiency does not restore absolute numbers of memory CD8$^+$ T cells during chronic bystander infection. (Related to Figure 7)

CD45.1 mice containing CD45.2 *Prdm1*$^{fl/fl}$ *Gzmb* Cre$^+$ **(A)**, *Tbet*$^{+/-}$ **(B)**, or littermate wt OT-1 cells were infected with VSV-OVA. On d8 p.i. OT-1 cells were isolated and adoptively transferred to “control” (naive) or “chronic LCMV” (d8 post-LCMV clone-13 infection). Numbers of OT-1 cells were analyzed on d30 post-transfer in spleen (n>7). Normal distribution was tested with D’Agostino & Pearson normality test. *NS*: not significant.

Supplemental Table 1. Gender and age of subjects used in the human cohorts. (Related to Figure 5)

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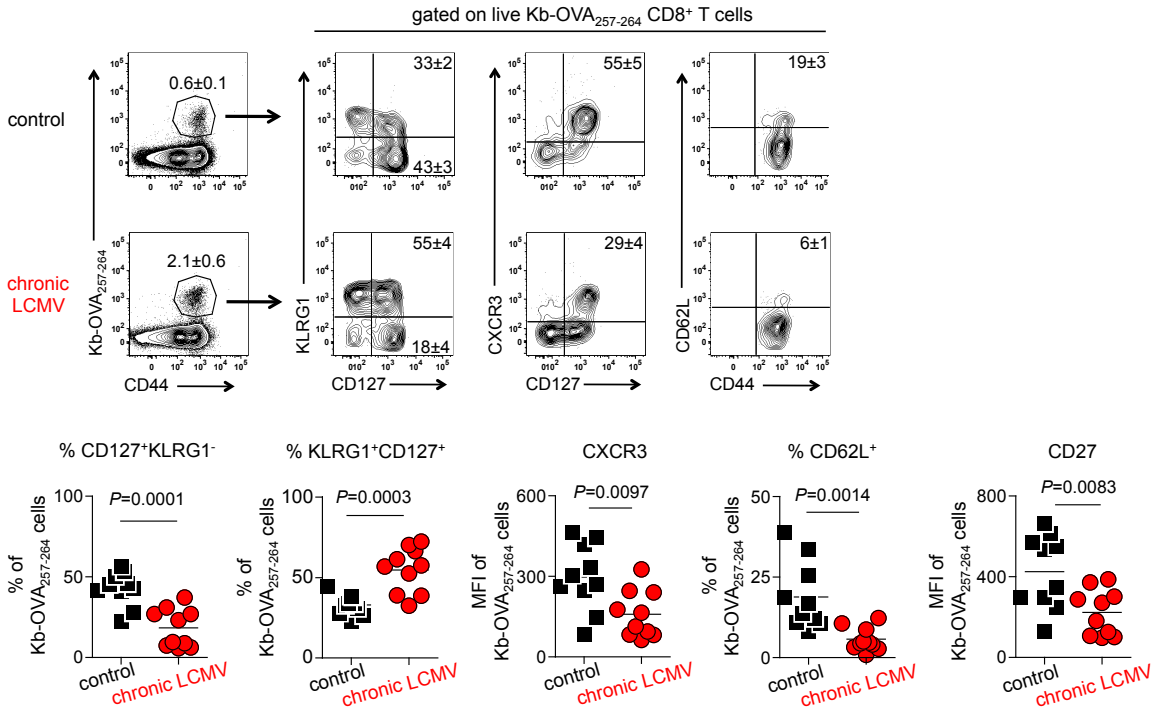
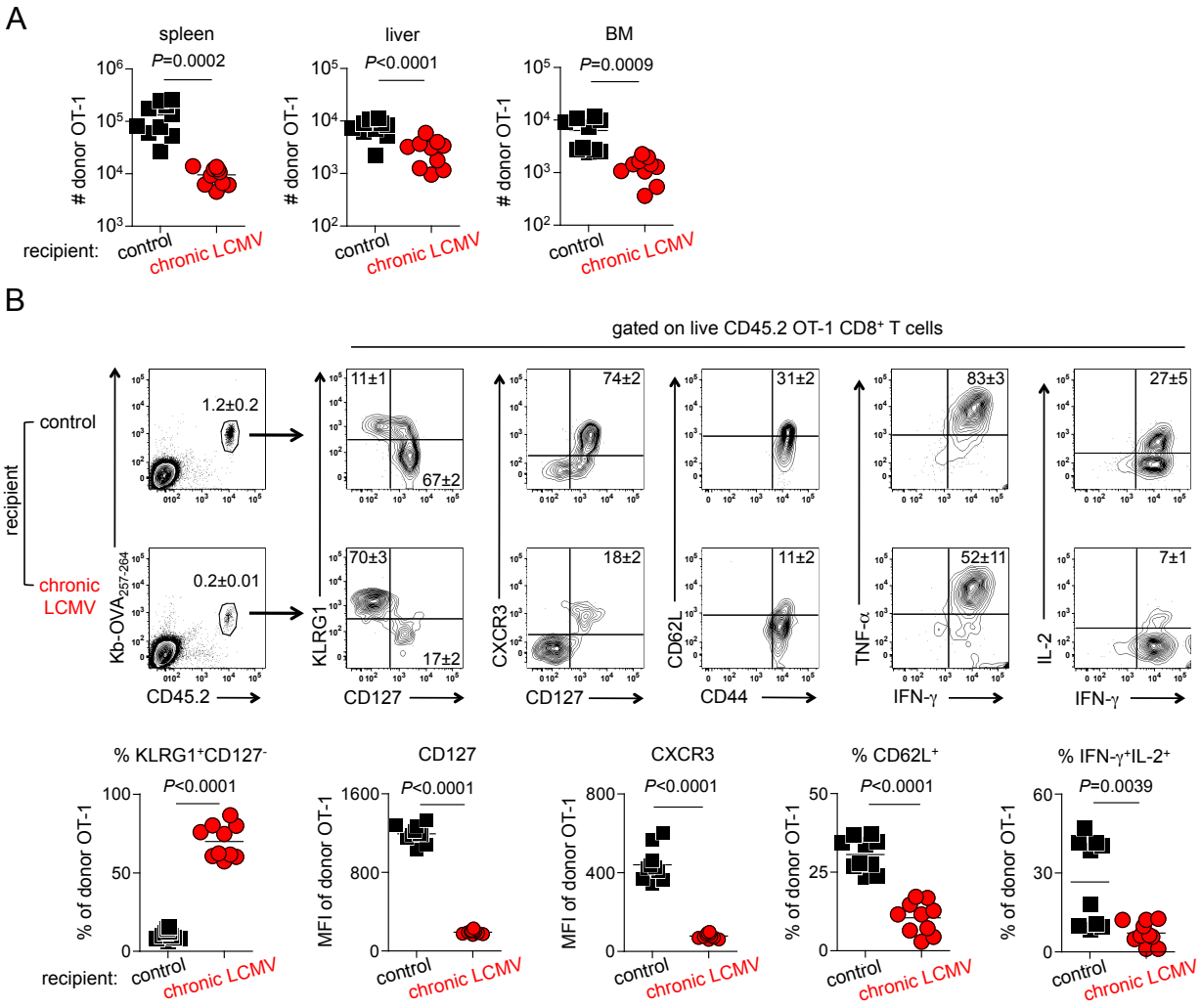


Stelekati et al., Supplemental Figure 1



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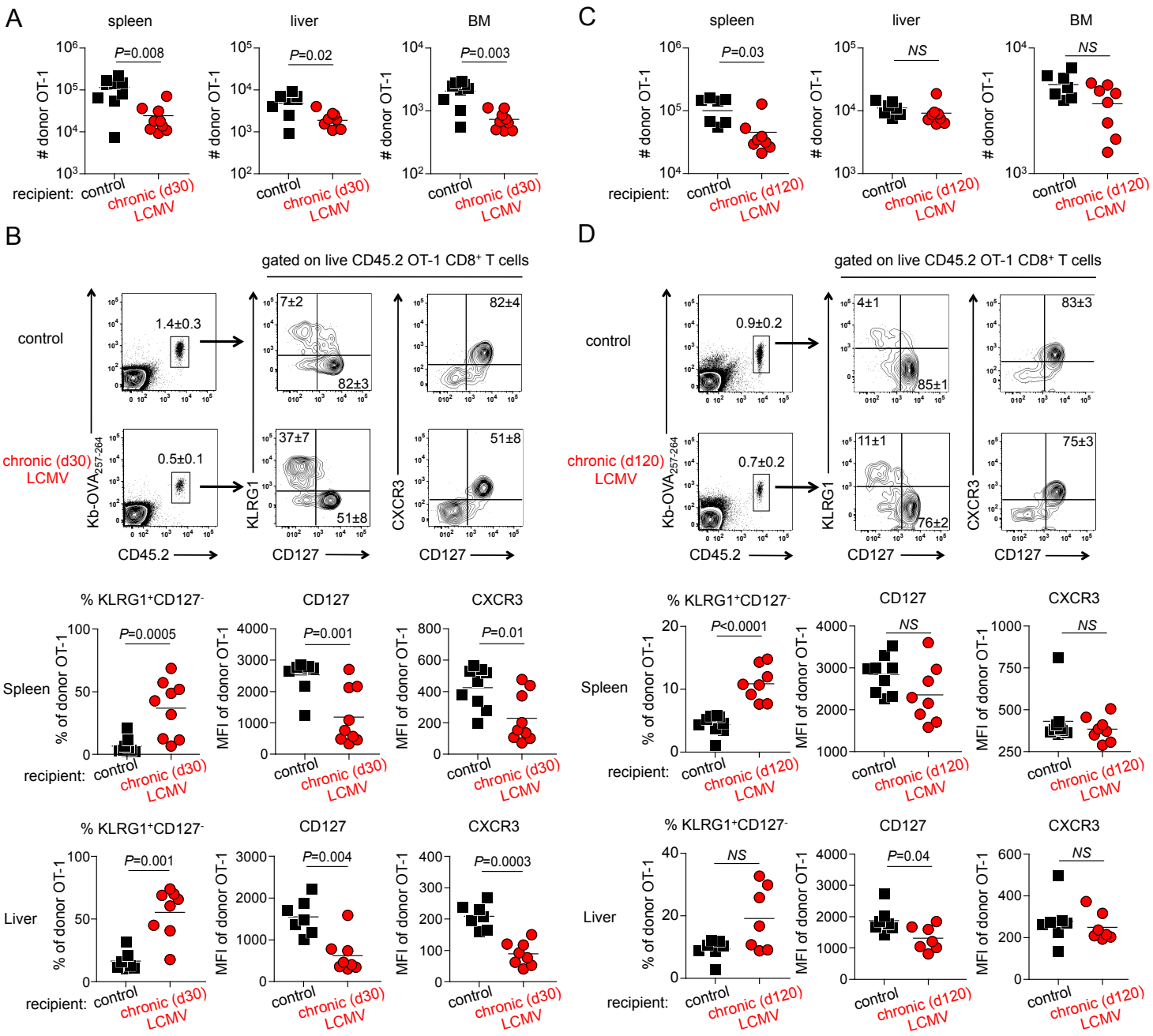

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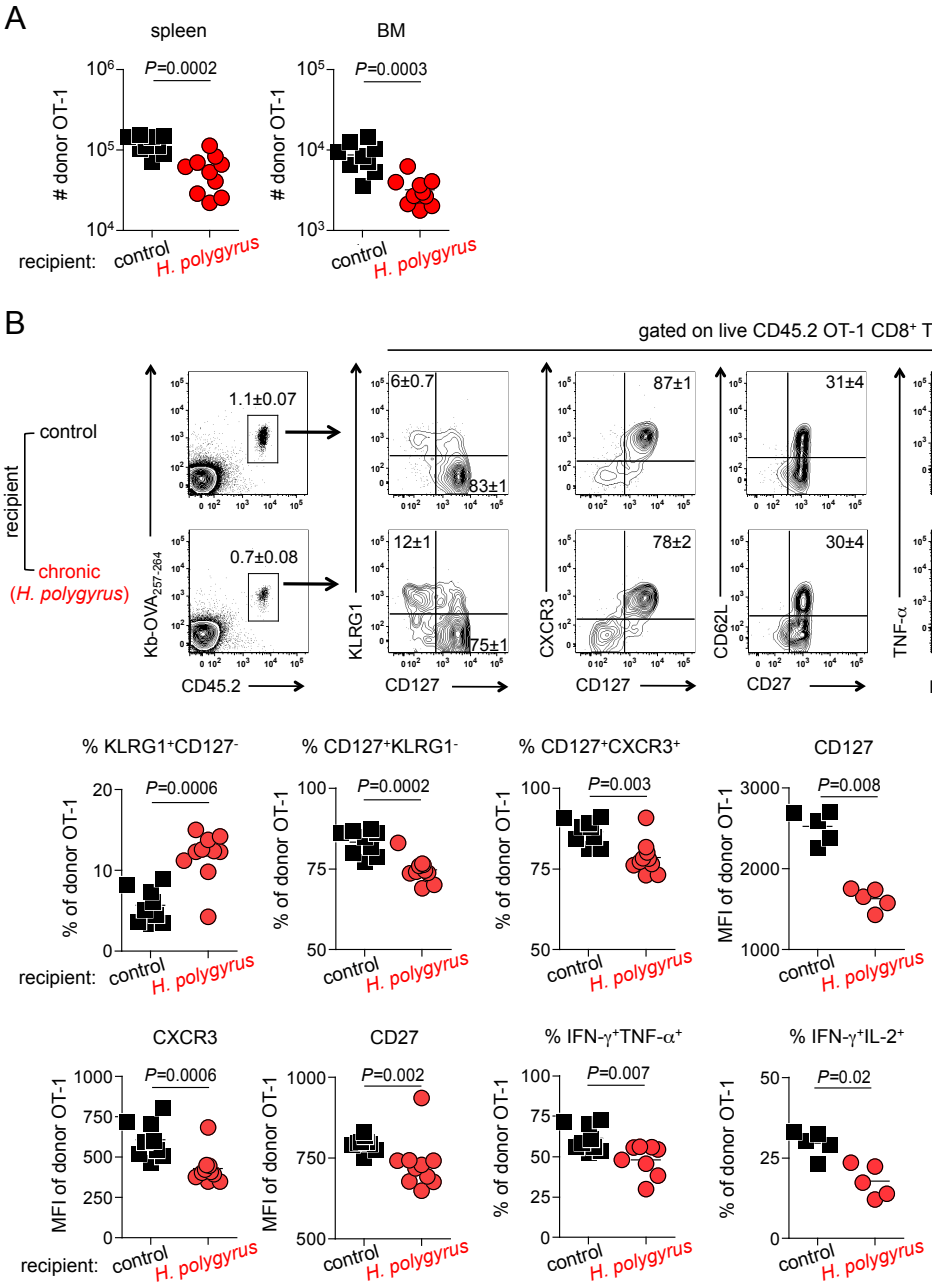

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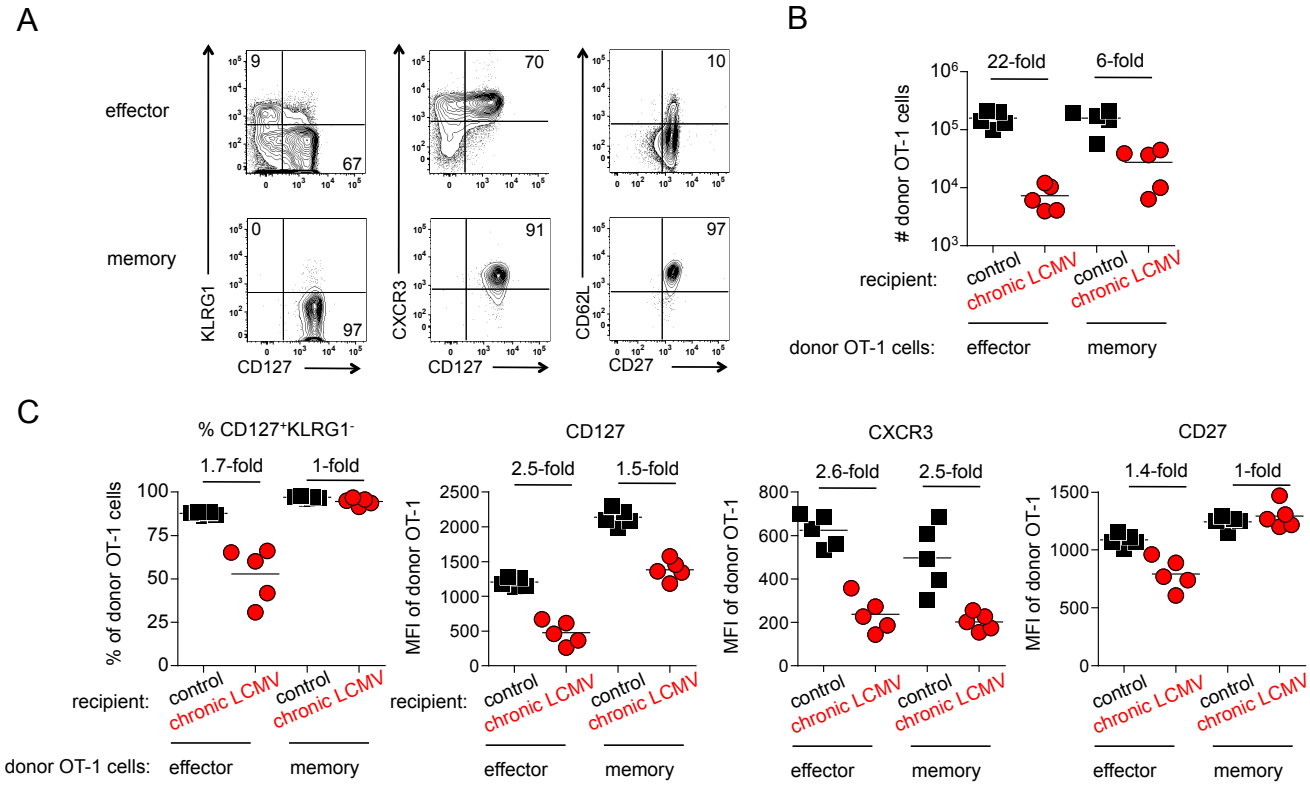

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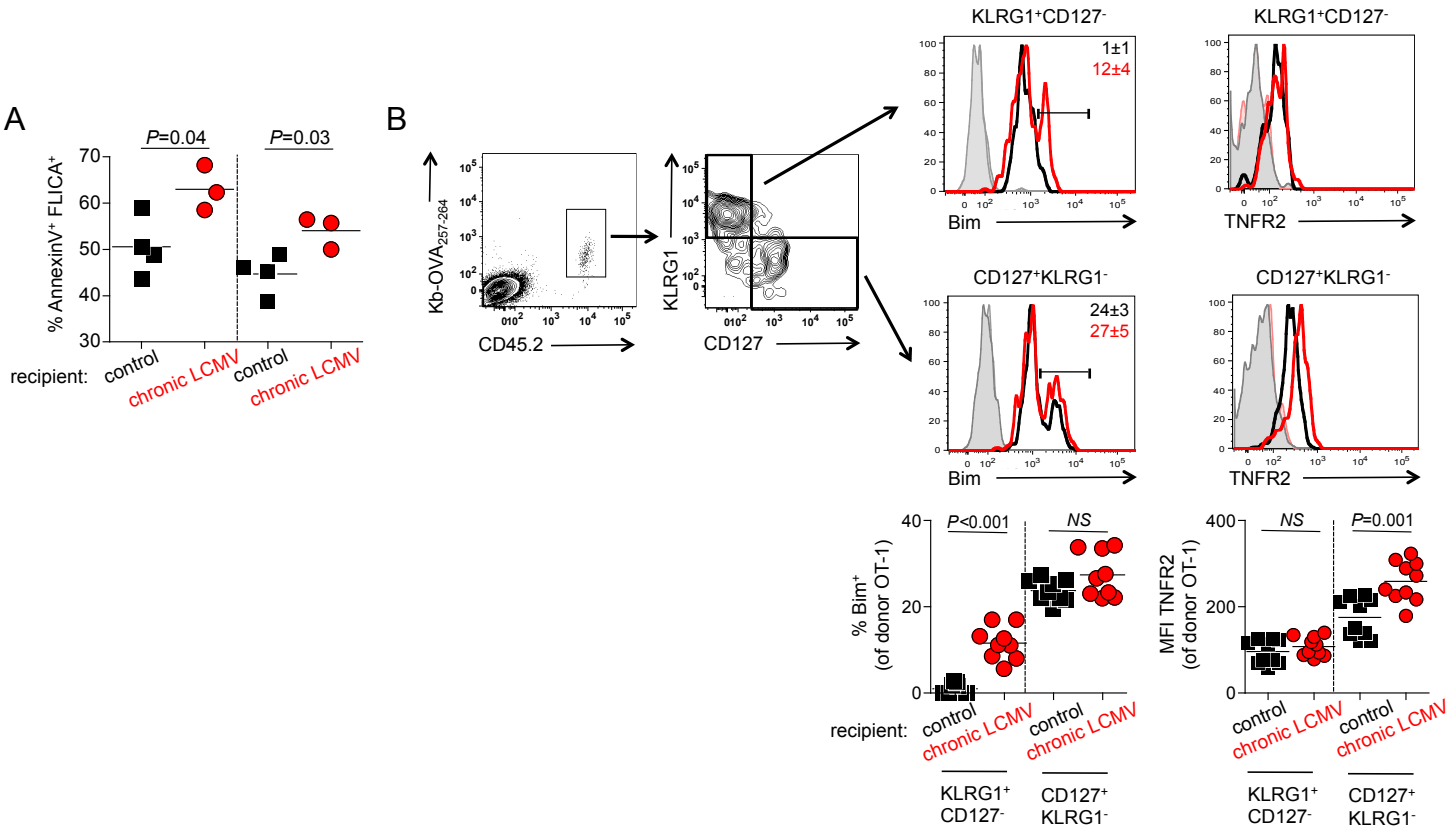

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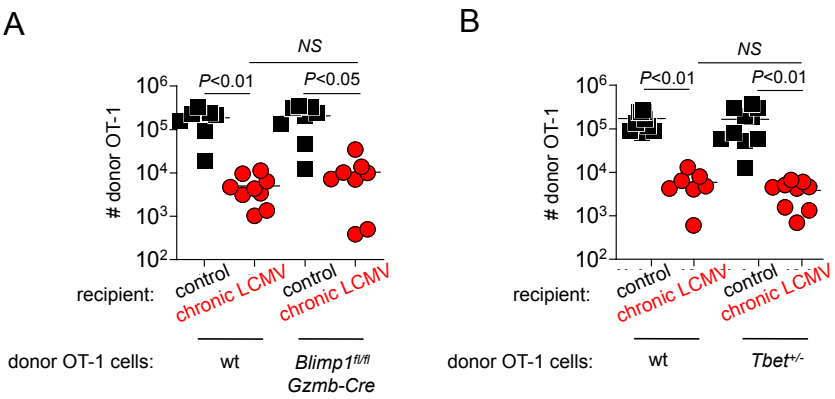

Stelekati et al., Supplemental Figure 7

	Subject ID	Gender	Age
Healthy subjects	1	M	24
	2	F	28
	3	F	27
	4	F	34
	5	F	37
	6	M	32
		67% female	**Mean age= 30**
HCV positive	7	M	20
	8	F	22
	9	F	42
	10	F	34
	11	M	39
	12	F	50
	13	F	34
		71% female	**Mean age= 34**

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