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Pathogen-Specific Inflammatory Milieux Tune the Antigen Sensitivity of CD8$^+$ T Cells by Enhancing T Cell Receptor Signaling

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SUMMARY

CD8$^+$ T cells confer host protection through T-cell-receptor (TCR)-mediated recognition of foreign antigens presented by infected cells. Thus, generation of CD8$^+$ T cell populations with high antigen sensitivity is critical for efficient pathogen clearance. Besides selection of high-affinity TCRs, the molecular mechanisms regulating the antigen sensitivity of CD8$^+$ T cells remain poorly defined. Herein, we have demonstrated that the antigen sensitivity of effector and memory CD8$^+$ T cells is dynamically regulated and can be tuned by pathogen-induced inflammatory milieux independently of the selection of cells with higher TCR affinity. Mechanistically, we have demonstrated that the signal-transduction capacity of key TCR proximal molecules is enhanced by inflammatory cytokines, which reduced the antigen density required to trigger antimicrobial functions. Dynamic tuning of CD8$^+$ T cell antigen sensitivity by inflammatory cytokines most likely optimizes immunity to specific pathogens while minimizing the risk of immunopathology at steady state.

INTRODUCTION

The capacity to clear infection relies on both the quantity and the quality of responding immune cells (Haring et al., 2006; Walker et al., 2010; Zhang and Bevan, 2011). Protection by CD8$^+$ T cells against intracellular pathogens strongly correlates with their ability to respond to low antigen density (high antigen sensitivity) (Alexander-Miller, 2005). Conversely, highly sensitive T cells could be detrimental for the host, resulting in immunopathology or autoimmunity (Amrani et al., 2000; Han et al., 2005). As such, the antigen sensitivity of CD8$^+$ T cells must be tightly regulated.

Although B cells enhance antigen sensitivity through somatic hypermutation of the B cell receptor, individual T cells are unable to directly change the binding affinity of their T cell receptor (TCR). However, increases in antigen sensitivity (also termed functional avidity [Slifka and Whitton, 2001]) occur during T cell responses (Busch and Pamer, 1999; Malherbe et al., 2004; Zehn et al., 2009). During infection or immunization, higher-avidity CD8$^+$ T cell clones, compared to lower-avidity clones, sustain proliferation, thus enhancing antigen sensitivity of the population (Zehn et al., 2009). Interestingly, monoclonal TCR transgenic (tg) CD8$^+$ T cells also increase their antigen sensitivity as they progress from early to late effector stages after infection (Slifka and Whitton, 2001). Thus, T cell intrinsic properties, unrelated to repertoire selection, can also control the antigen sensitivity of CD8$^+$ T cells. Together, these studies underscore the possibility that antigen sensitivity of individual CD8$^+$ T cells might be dynamically regulated independently of changes in TCR utilization. However, the molecular mechanisms regulating antigen sensitivity of effector CD8$^+$ T cells remain poorly understood. Additionally, it remains unknown whether functional avidity maturation is hardwired in cells with a specific TCR (Slifka and Whitton, 2001) or whether antigen sensitivity can be modulated by pathogen-specific signals.

Inflammatory cytokines provide an additional signal that, along with TCR ligation and costimulation, is required for maximal effector CD8$^+$ T cell accumulation (Curtsinger and Mescher, 2010; Curtsinger et al., 1999; Curtsinger et al., 2005). The inflammatory cytokines permitting optimal CD8$^+$ T cell responses depend on the inflammatory milieu induced by the invading pathogen (Aichele et al., 2006; Kolumam et al., 2005; Pearce and Shen, 2007). The inflammatory environments induced by infection also sustain effector differentiation and delay the formation of functional memory CD8$^+$ T cells (Badovinac et al., 2005; Haring et al., 2006; Joshi et al., 2007; Joshi and Kaech, 2008; Pham et al., 2009). In contrast, immunization with peptide-coated mature dendritic cells (DCs) in the absence of systemic inflammation induces CD8$^+$ T cells that rapidly acquire memory characteristics (Badovinac et al., 2005; Pham et al., 2009). Given that the inflammatory milieu plays multiple roles in regulating the T cell response, we tested the hypothesis that inflammatory cytokines directly regulate antigen sensitivity of CD8$^+$ T cells independently of clonal selection.

RESULTS

Infection-Induced Inflammatory Milieux Regulate Antigen Sensitivity of Endogenous Effector CD8$^+$ T Cells

To determine whether the inflammatory milieu regulates the antigen sensitivity of effector CD8$^+$ T cells, we immunized mice

with lipopolysacharide (LPS)-matured DCs coated with ovalbumin$_{257–264}$ (OVA) peptide (DC-OVA) in the presence or absence of infection with attenuated *Listeria monocytogenes* (LM) or lymphocytic choriomeningitis virus strain Armstrong (LCMV). Infection with LM or LCMV, which do not express OVA, induces multiple inflammatory cytokines (Biron, 1999; Pamer, 2004). This system allowed us to study the effect of the pathogen-specific inflammatory milieu in a setting where antigen presentation remains constant (Badovinac et al., 2005; Pham et al., 2009).

Antigen sensitivity of endogenous OVA-specific CD8$^+$ T cells was measured on day 5 after DC immunization by ex vivo stimulation with titrated doses of OVA peptide, and the cells were subsequently intracellularly stained for effector cytokines. We evaluated antigen sensitivity on day 5 because pathogen clearance occurs before peak CD8$^+$ T cell numbers and because the effector function of CD8$^+$ T cells at early time points is therefore critical to an effective immune response (Badovinac et al., 2002; Pope et al., 2001). We observed that infection with either LM or LCMV significantly ($p < 0.01$ and $p < 0.001$, respectively) increased the antigen sensitivity of the responding OVA-specific CD8$^+$ T cells, as determined by the percentage of cells producing interferon-γ (IFN-γ) (Figures 1A and 1B). Compared with that of OVA-specific CD8$^+$ T cells from mice immunized with only DC-OVA, the antigen sensitivity of OVA-specific CD8$^+$ T cells was increased 2.9-fold with LM infection and 10.7-fold with LCMV infection (Figure 1B), as measured by the peptide concentration required for obtaining 50% of the maximum IFN-γ production (effective concentration 50 [EC$_{50}$]) (Figure 1B). In addition, exposure to inflammation significantly ($p < 0.01$ and $p < 0.001$ for LM and LCMV, respectively) increased IFN-γ production per cell (Figure 1C and Figure S1A, available online). Thus, the inflammatory milieu induced by infection enhanced antigen sensitivity of OVA-specific effector CD8$^+$ T cells. The inflammatory milieu associated with LCMV infection resulted in a higher sensitivity to antigen than did LM infection. Thus, the antigen sensitivity of early effector CD8$^+$ T cells was tuned in a pathogen-specific manner.

Antigen Sensitivity Can Be Regulated Independently of Clonal Selection

The preceding analyses of endogenous effector CD8$^+$ T cells did not distinguish whether enhanced antigen sensitivity depends on the selection of high-affinity T cells. To address this, we sought to determine whether infection-associated inflammation increases antigen sensitivity of monoclonal TCR tg CD8$^+$ T cells. Physiologic numbers of Thy1.1/1.1 or Thy1.1/1.2 OVA-specific OT-I TCR tg CD8$^+$ T cells were adoptively transferred into Thy1.2/1.2 mice. We immunized recipients 24 hr later with DC-OVA in the presence or absence of LM or LCMV infection to induce pathogen-specific inflammation. To control for potential effects of infection on the function of antigen-presenting cells (APCs) during ex vivo stimulation, we stimulated congenically marked OT-I cells from the DC-OVA-only group in the same well as OT-I cells from mice immunized with DC-OVA in the presence of LM or LCMV infection. As a result of the fixed TCR and common APC pool in this scenario, any observed differences in antigen sensitivity between groups must be T cell intrinsic. We observed that infection with LM or LCMV also significantly ($p < 0.05$ and $p < 0.001$, respectively) increased the antigen sensitivity of monoclonal OT-I CD8$^+$ T cells on day 5 after immunization (Figures 1D and 1E). Measured by the EC$_{50}$ of peptide required for IFN-γ production, the antigen sensitivity of OT-I CD8$^+$ T cell IFN-γ production was 2.4-fold higher with LM infection and 7.6-fold higher with LCMV infection than that of OT-I CD8$^+$ T cells generated with DC-OVA alone (Figure 1E). Similarly, LM and LCMV infection enhanced the antigen sensitivity for the production of OT-I tumor necrosis factor α (TNF-α) (Figures S1C and S1D). This increase in antigen sensitivity permitted robust cytokine production at antigen concentrations that were insufficient to induce a cytokine response from OT-I CD8$^+$ T cells generated by DC-OVA immunization alone. As observed with endogenous responses, LCMV infection exerted a greater effect than did LM infection on OT-I CD8$^+$ T cell antigen sensitivity and the per cell production of IFN-γ (Figures 1E and 1F and Figure S1B). These data show that the pathogen-specific inflammatory milieu regulates antigen sensitivity of CD8$^+$ T cells for effector-cytokine production independently of clonal selection or APC function.

Inflammatory cytokines are rapidly produced after infection and could exert their effects by altering priming of naive CD8$^+$ T cells. To address this, we infected mice with LCMV at day 3 after DC-OVA immunization, a time point when priming of new T cells by DC immunization is not detectable (Badovinac et al., 2005; Prlic et al., 2006). Infection with LCMV on day 3 postimmunization still significantly enhanced ($p = 0.015$) the antigen sensitivity of OT-I CD8$^+$ T cells for IFN-γ production at day 5 by 5.6-fold, as measured by EC$_{50}$ (Figures 1G and 1H). Thus, the infection-induced inflammatory milieu rapidly influences antigen sensitivity of CD8$^+$ T cells independently of the effects on priming.

We next determined whether inflammation simply accelerates acquisition of peak antigen sensitivity by CD8$^+$ T cells or also enhances antigen sensitivity for prolonged periods. Infection with LM or LCMV resulted in significantly higher antigen sensitivity ($p = 0.0003$ and $p = 0.004$, respectively) and per cell IFN-γ production ($p = 0.0027$ and $p = 0.0019$, respectively) in OT-I CD8$^+$ T cells on day 8 postimmunization than in OT-I CD8$^+$ T cells generated by DC-OVA alone (Figure 2 and Figure S2). Comparison of the EC$_{50}$ values for IFN-γ production by OT-I cells in the presence of LCMV infection revealed lower antigen sensitivity at day 8 (EC$_{50}$ = 44 pM) (Figure 2E) than at day 5 (EC$_{50}$ = 21 pM) (Figure 1F). Thus, the antigen sensitivity of CD8$^+$ T cells exposed to a pathogen-specific inflammatory milieu can be dynamically regulated to peak early after infection and then decrease as the infection is resolved.

Infection-Induced Inflammatory Milieu Enhances Cytolysis by CD8$^+$ T Cells

In addition to effector cytokines, CD8$^+$ T cells are cytolytic through the perforin and granzyme B granule-exocytosis pathways. OT-I CD8$^+$ T cells from mice immunized with DC-OVA and LM or LCMV expressed more granzyme B (Figure 3A) and also exhibited a decreased EC$_{50}$ of OVA peptide required for degranulation (as measured by surface CD107a expression, Figures 3B and 3C). Consistent with these data, OT-I T cells primed in mice receiving LCMV infection exhibited significantly ($p = 0.026$ and $p = 0.023$) enhanced per cell in vivo killing of

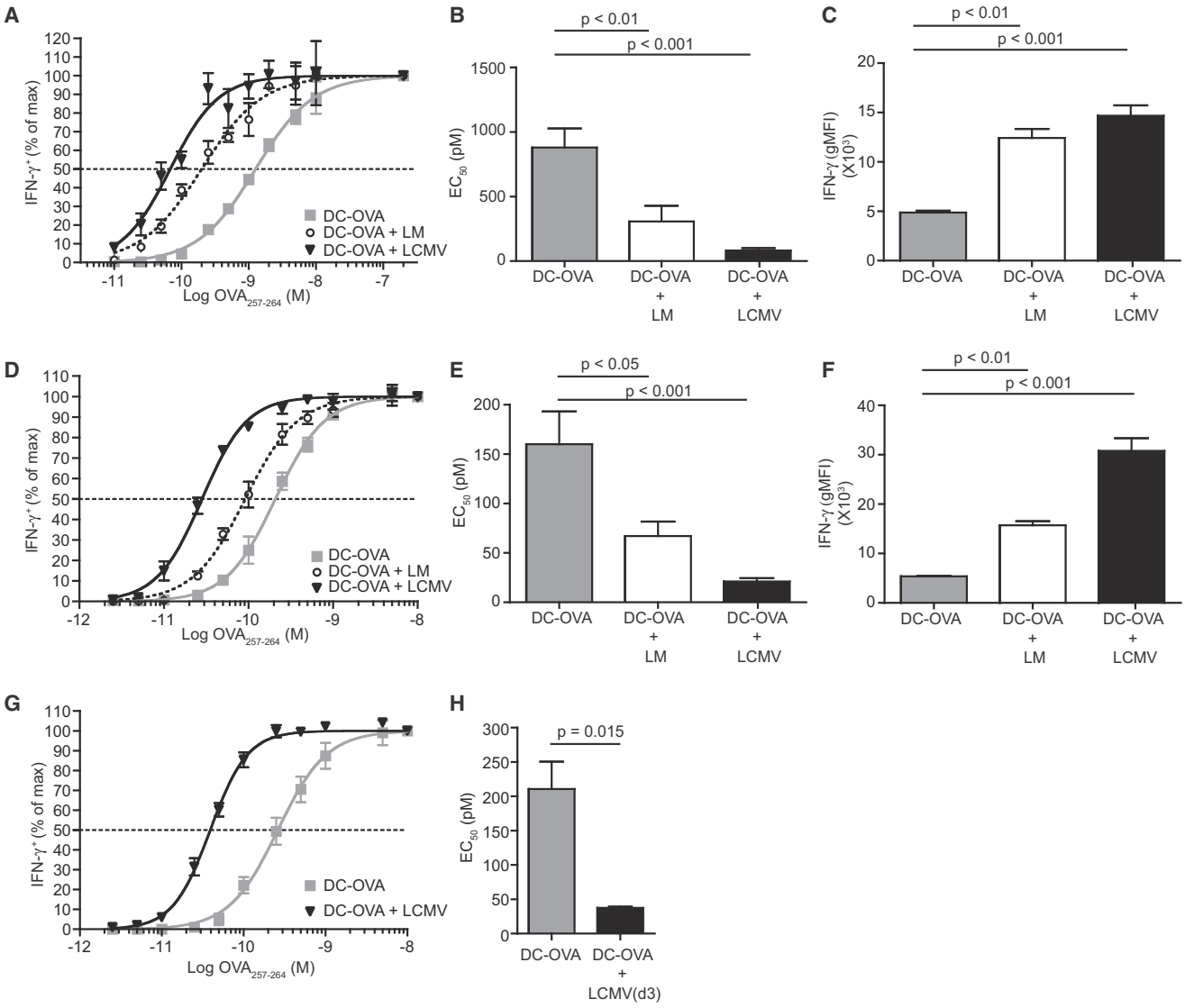

Figure 1. Pathogen-Specific Inflammation Increases Antigen Sensitivity of Effector CD8⁺ T Cells

(A) The percentage of IFN-γ⁺ endogenous CD8⁺ T cells at day 5 after immunization with DC-OVA alone (gray squares) or DC-OVA coinfected with LM (open circles) or LCMV (black triangles) was determined after ex vivo stimulation with titrated concentrations of OVA peptide. Data (mean ± SEM) are normalized to the percentage of IFN-γ⁺ cells at peptide saturation (200 nM).

(B) Summary (mean ± SEM) of EC_{50} for stimulation of IFN-γ production by endogenous OVA-specific CD8⁺ T cells.

(C) Geometric mean fluorescence intensity (gMFI) (mean ± SEM) of IFN-γ by endogenous OVA-specific CD8⁺ T cells at peptide saturation (200 nM).

(D–F) The same as (A)–(C), respectively, but for OT-I CD8⁺ T cells (saturating peptide = 10 nM).

(G and H) The same as (A) and (B), respectively, but for OT-I CD8⁺ T cells after LCMV infection on day 3 after immunization with DC-OVA.

Data in (A), (C), (D), (F), and (G) are from three mice per group and are representative of at least two independent experiments. Data in (B), (E), and (H) are cumulative from two to three independent experiments with at least six mice per group. Data in (B), (C), (E), and (F) were analyzed by one-way ANOVA with Tukey's post-test of multiple comparisons. Data in (H) were analyzed with a two-tailed, unpaired Student's t test. See also Figure S1.

splenocytes coated with two doses of OVA peptide (Figures 3D and 3E). Thus, the inflammatory milieu regulates the in vivo capacity of effector CD8⁺ T cells to kill targets displaying low amounts of antigen.

Inflammation Enhances TCR Signaling

To address underlying mechanisms of inflammation-induced T-cell-intrinsic increases in antigen sensitivity, we initially ruled out changes in surface expression of the alpha (Vα2) and beta (Vβ5) chains of the TCR or CD3ε, the costimulatory molecule CD28, or CD11a (the α chain of LFA-1, a critical integrin that facilitates conjugation between T cells and DCs [Dustin et al., 1989]) (Figure 4A). However, we did observe that the sensitivity of IFN-γ production to plate-bound CD3 antibody was 5.3-fold higher in OT-I cells primed in the presence of LCMV-induced inflammation than in OT-I cells immunized with DC-OVA alone

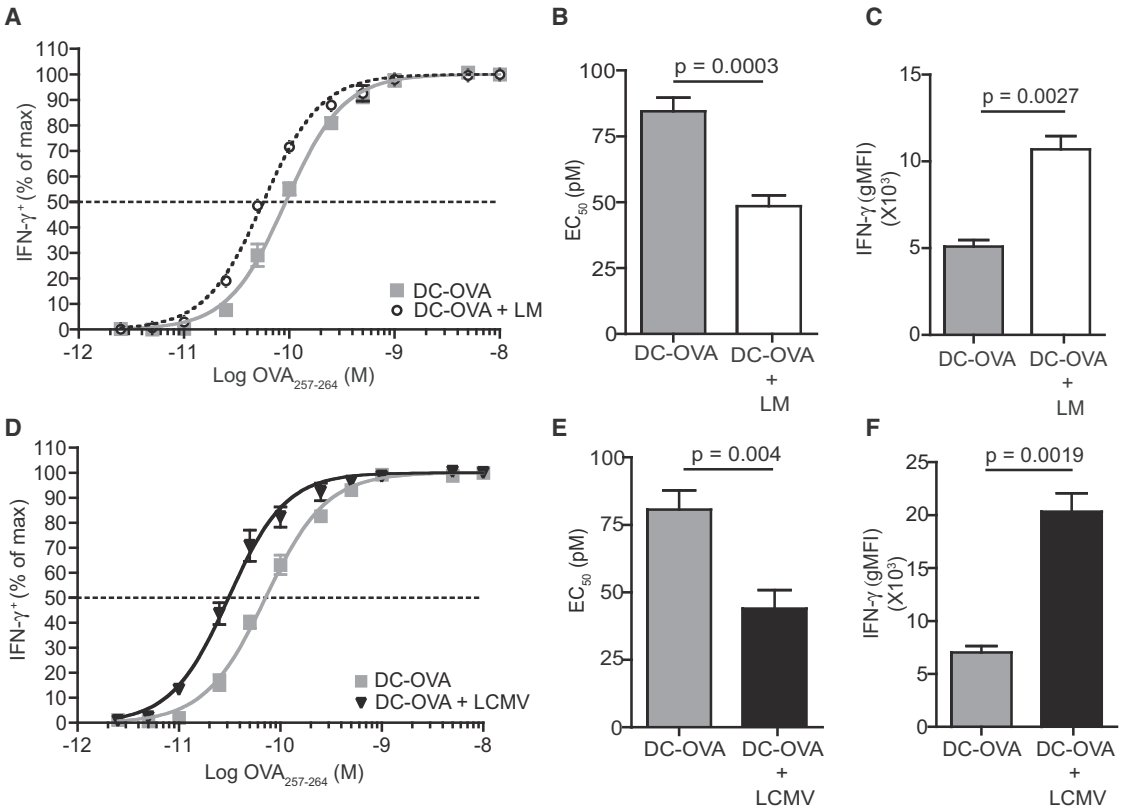

Figure 2. Infection-Induced Inflammation Enhances Antigen Sensitivity of CD8$^+$ T Cells for Several Days

(A) The percentage of IFN-γ$^+$ OT-I CD8$^+$ T cells at day 8 after immunization with DC-OVA alone (gray square) or DC-OVA and LM (open circles) was determined after ex vivo stimulation with titrated concentrations of OVA peptide. Data are normalized as in Figure 1 except that peptide saturation was 10 nM.

(B) Summary (mean ± SEM) of EC_{50} for stimulation of IFN-γ production by OT-I CD8$^+$ T cells.

(C) gMFI (mean ± SEM) of IFN-γ expressed by OT-I CD8$^+$ T cells at peptide saturation (10nM).

(D–F) The same as (A)–(C), respectively, but for OT-I CD8$^+$ T cells on day 8 after immunization with DC-OVA alone (gray squares) or DC-OVA and LCMV (black triangles).

Data in (A), (C), (D), and (F) are from three mice per group and are representative of two independent experiments. Data in (B) and (E) are cumulative from two independent experiments with at least six mice per group. Data were analyzed with a two-tailed, unpaired Student's t test. See also Figure S2.

(Figure 4B). These data suggest that inflammation regulates antigen sensitivity by increasing the capacity to translate TCR signals rather than by enhancing APC or costimulatory interactions.

Ligation of the TCR results in the activation of a signaling cascade initiated by the tyrosine kinases LCK and ZAP-70. Studies have suggested that enhanced antigen sensitivity following infection might be regulated by increased expression of these key signaling molecules (Amoah et al., 2012; Slifka and Whitton, 2001). However, exposure to inflammation did not increase the protein expression of either of these kinases at day 5 postimmunization (as measured by the relative signal intensity compared to the β-actin loading control) (Figures 4C and 4D and Figures S3A and S3B). Therefore, we assessed whether inflammation increased antigen sensitivity of CD8$^+$ T cells by enhancing their capacity to translate TCR signals. CD3-antibody stimulation resulted in enhanced and more rapid phosphorylation of ZAP-70 in CD8$^+$ T cells exposed to inflammation than in OT-I CD8$^+$ T cells from mice immunized with DC-OVA alone (Figure 4D and Figure S3C). Thus, CD8$^+$ T cells exposed to inflammation exhibit enhanced activation of ZAP-70 after TCR stimulation, which occurs independently of changes in total LCK or ZAP-70.

Phosphorylation of ZAP-70 leads to the activation of phospholipase C γ (PLC)-γ, which cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2) into the secondary messengers inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). These secondary messengers initiate intracellular calcium release and the activation of protein kinase C (PKC) and the mitogen-activated protein kinase (MAPK) pathway, resulting in the transcription of target genes, including IFN-γ (Abraham and Weiss, 2004; Smith-Garvin et al., 2009). We observed an increase in the phosphorylation of PLC-γ on activating tyrosine 783 in CD8$^+$ T cells exposed to inflammation (Figure 4E and Figure S3D), demonstrating that exposure to inflammation enhanced the activation of signaling pathways downstream of TCR ligation. This led to dramatic increases in the activation of PLC-γ-dependent MAPK pathways (Nolz et al., 2006), as shown by the marked enhancement in the phosphorylation of extracellular-signal-regulated kinases 1 (ERK1) and 2 (ERK2) and

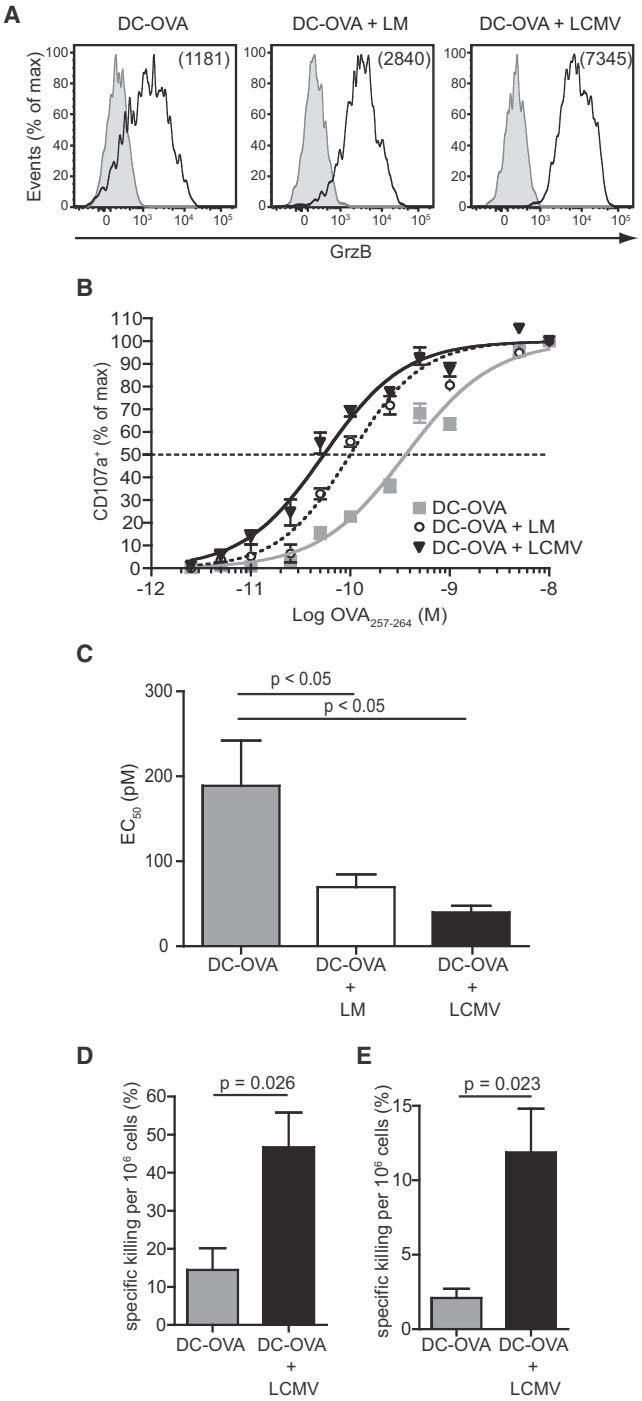

Figure 3. Infection-Induced Inflammation Enhances Cytolysis by CD8$^+$ T Cells

(A) Representative plots of granzyme B expression at day 5 after DC-OVA immunization with or without LM or LCMV infection. Shaded histograms are isotype controls. Numbers show gMFI of granzyme B staining. Data are representative of at least three mice per group and two independent experiments.

(B) The percentage of CD107a$^+$ OT-I CD8$^+$ T cells at day 5 after immunization with DC-OVA alone (gray square) or DC-OVA coinfected with LM (open circles) or LCMV (black triangles) was measured after ex vivo stimulation with titrated concentrations of OVA peptide. Data (mean ± SEM) are normalized as in Figure 1 except that peptide saturation was 10 nM. Data are from three mice per group and are representative of two independent experiments.

(C) Summary (mean ± SEM) of EC_{50} for degranulation (CD107a staining) of OT-I CD8$^+$ T cells. Data are cumulative from two independent experiments with a total of six mice per group and were analyzed by one-way ANOVA with Tukey's post-test of multiple comparisons.

(D) The percentage of in-vivo-specific killing of splenocytes coated with 0.05 nM OVA peptide. Data are the percentage of killing for 1 × 10^6 OT-I CD8$^+$ T cells in recipient mice. Data are from at least four mice per group, are representative of two independent experiments, and were analyzed with a two-tailed, unpaired Student's t test.

(E) The same as (D) but for splenocytes coated with 0.025 nM OVA peptide.

c-Jun N-terminal kinases 1 (JNK1) and 2 (JNK2) in CD3-stimulated CD8$^+$ T cells exposed to inflammation (Figures 4F and 4G and Figures S3E and S3F). We did not observe enhanced phosphorylation of another MAPK, p38 (Figures S3G and S3H). In T cells, p38 can be activated in a noncanonical manner independent of linker of activated T cells (LAT) (Salvador et al., 2005). These data suggest that exposure to inflammation specifically enhances the canonical MAPK activation pathway. In contrast, we saw no differences in ERK1 and ERK2 activation when cells were stimulated with phorbol myristate acetate (PMA), a DAG analog that activates the MAPK pathway and phosphorylation of ERK1 and ERK2 while bypassing the requirement for TCR ligation and proximal signaling events (Figure 4H). Thus, although CD8$^+$ T cells stimulated with DC-OVA in a noninflammatory milieu were capable of robust MAPK activation, exposure of these cells to pathogen-induced inflammation enhanced TCR proximal signaling, amplified MAPK activation, and increased antigen sensitivity.

To determine whether the enhanced TCR signaling controlled antigen sensitivity in cells exposed to inflammation, we evaluated IFN-γ production from cells stimulated with either OVA peptide or PMA and ionomycin. We observed a larger fold increase in per cell IFN-γ production by OT-I T cells exposed to inflammation after peptide stimulation than in OT-I T cells exposed to inflammation after stimulation with PMA and ionomycin (Figure 4I). These data confirm that exposure to inflammation increases antigen sensitivity by enhancing proximal TCR signaling.

Direct Regulation of Antigen Sensitivity by Inflammatory Cytokines

Pathogens elicit distinct and complex inflammatory-cytokine responses. For example, LM infection induces several cytokines, including interleukin-12 (IL-12) and type 1 interferon, whereas LCMV elicits a dominant type 1 interferon response (Biron, 1999; Pamer, 2004). As such, we sought to determine whether regulation of antigen sensitivity by exposure to inflammation requires direct signaling to CD8$^+$ T cells by specific cytokines. Congenically marked wild-type (WT) OT-I CD8$^+$ T cells and OT-I CD8$^+$ T cells deficient in IL-12 receptor β1 (*Il12rb1*$^{-/-}$) were cotransferred into WT mice, which were immunized with DC-OVA in the presence or absence of infection with LM or LCMV. Compared to WT OT-I CD8$^+$ T cells, *Il12rb1*$^{-/-}$ OT-I CD8$^+$ T cells responded to immunization with DC-OVA alone and did not exhibit changes in antigen sensitivity (Figure 5A). However, antigen sensitivity of *Il12rb1*$^{-/-}$ OT-I CD8$^+$ T cells in mice receiving LM infection was significantly ($p = 0.004$) lower

than that of WT OT-I CD8$^+$ T cells (Figure 5B). Thus, IL-12 signaling directly regulates the antigen sensitivity of CD8$^+$ T cells. Despite this, the LM-induced inflammatory milieu still partially increased the antigen sensitivity of *Il12rb1*$^{-/-}$ OT-I CD8$^+$ T cells (Figures 5A and 5B), indicating that IL-12 is not the only cytokine involved in modulating the antigen sensitivity of responding CD8$^+$ T cells after LM infection. *Il12rb1*$^{-/-}$ and WT OT-I cells exhibited the same enhancement of antigen sensitivity after DC-OVA immunization in the presence of LCMV infection (which primarily drives type 1 interferon and not IL-12) (Figure 4C).

To address a role for type 1 interferon signaling and extend our results to an additional TCR tg CD8$^+$ T cell, we cotransferred congenically marked WT P14 (specific to the GP_{33-41} epitope of LCMV) CD8$^+$ T cells and P14 CD8$^+$ T cells deficient in type 1 interferon receptor (*Ifnar1*$^{-/-}$) into WT mice, which were immunized with DC-GP33 and subsequently infected with LM or Pichinde virus (PV) on day 3 postimmunization. PV is an arenavirus that does not express the GP_{33-41} epitope of LCMV. Infection with either LM or PV enhanced the antigen sensitivity of WT P14 CD8$^+$ T cells (Figures 5D–5F), whereas the absence of the type 1 interferon receptor strongly abrogated the enhanced antigen sensitivity of P14 CD8$^+$ T cells in PV-infected mice (Figure 5F). Thus, direct type 1 interferon signaling on CD8$^+$ T cells plays a major role in the enhanced antigen sensitivity associated with the exposure to PV-induced inflammation. Deficiency of type 1 interferon receptor did not affect the capacity of LM to enhance the antigen sensitivity of P14 CD8$^+$ T cells, suggesting that although type 1 interferons are induced by LM infection (Pamer, 2004), these cytokines do not play a dominant role in regulating antigen sensitivity over the course of this bacterial infection. Collectively, these data demonstrate that inflammatory cytokines directly regulate the antigen sensitivity of effector CD8$^+$ T cells in a pathogen-specific manner.

To determine whether direct cytokine signaling on CD8$^+$ T cells causes enhanced TCR signaling, we cotransferred congenically marked WT and *Ifnar1*$^{-/-}$ P14 CD8$^+$ T cells into WT mice and immunized them with DC-GP33 with or without infection with PV on day 3 after DC-GP33 immunization. Two days later, phosphorylation of ERK1 and ERK2 after CD3 stimulation was analyzed by flow cytometry, which allowed us to compare WT and *Ifnar1*$^{-/-}$ T cells immunized in the same mice. As described for WT OT-I CD8$^+$ T cells (Figure 4), exposure to inflammation enhanced ERK1 and ERK2 phosphorylation after stimulation of WT P14 CD8$^+$ T cells (Figures 5G and 5H). Enhanced ERK1 and ERK2 activation was abrogated in *Ifnar1*$^{-/-}$ P14 cells (Figures 5G and 5H). ERK1 and ERK2 phosphorylation in *Ifnar1*$^{-/-}$ P14 cells was similar to that in WT P14 cells when proximal TCR signaling was bypassed by PMA stimulation, demonstrating that *Ifnar1*$^{-/-}$ cells retain the capacity to signal normally (Figure 5H). These data demonstrate that inflammatory cytokines act directly on effector CD8$^+$ T cells to enhance proximal TCR signaling and increase antigen sensitivity.

Inflammatory Cytokines Enhance Antigen Sensitivity of Memory CD8$^+$ T Cells

Hosts will contain memory CD8$^+$ T cells specific to multiple pathogens, and these cells enhance immunity to pathogen re-exposure. To determine whether inflammatory cytokines also enhance antigen sensitivity of memory CD8$^+$ T cells, we infected mice containing memory OT-I or P14 CD8$^+$ T cells with an unrelated pathogen to induce inflammatory cytokines in the absence of antigen stimulation (LCMV or PV, respectively). On day 4 after infection, we observed that exposure to inflammatory cytokines significantly increased the antigen sensitivity of memory OT-I CD8$^+$ T cells (Figures 6A and 6B) and memory P14 CD8$^+$ T cells (Figures 6C and 6D) ($p = 0.0026$ and $p = 0.0017$, respectively), as well as the per cell IFN-γ production (Figure S3). Thus, antigen sensitivity of memory CD8$^+$ T cells was also regulated by inflammatory cytokines. This change does not require recent TCR stimulation, suggesting that inflammatory cytokines enhance antigen sensitivity of memory cells prior to their potential interaction with cells expressing cognate antigen.

Chemokines induced by tissue-specific inflammation mediate rapid, antigen-independent recruitment of memory CD8$^+$ T cells (Ely et al., 2003; Kohlmeier et al., 2008). To determine whether tissue-specific inflammation enhances antigen sensitivity, we infected mice harboring memory OT-I CD8$^+$ T cells intranasally with LCMV to induce inflammatory cytokines and recruitment of memory cells to the lung. On day 3 after infection, lung OT-I cells exposed to inflammation exhibited significantly ($p = 0.0097$) increased antigen sensitivity (Figure 6E), indicating that exposure to inflammatory cytokines regulated the antigen sensitivity of memory cells localized within the inflamed tissue.

Given that inflammatory cytokines tuned the antigen sensitivity of memory CD8$^+$ T cells independently of antigen re-encounter, we next sought to determine whether tuning is dynamic and antigen sensitivity returns to steady state after clearance of infection. Mice containing memory OT-I CD8$^+$ cells were mock infected or infected with LCMV either 14 days or 4 days before the analysis such that antigen sensitivity in all groups could be measured on the same day. Although OT-I cell antigen sensitivity was significantly ($p < 0.05$) enhanced on day 4 postinfection, this increase was transient because antigen sensitivity was no longer significantly different from mock-infected mice on day 14 after LCMV infection (Figure 6F). Thus, tuning of memory CD8$^+$ T cell antigen sensitivity is dynamic, and in the absence of antigen re-encounter, antigen sensitivity of bystander memory CD8$^+$ T cells returns to steady state.

Inflammatory Cytokines Enhance TCR Signaling of Memory CD8$^+$ T Cells

To determine whether inflammatory cytokines increase antigen sensitivity by enhancing TCR signaling, we purified memory OT-I TCR tg CD8$^+$ T cells on day 4 after LCMV infection or mock infection and stimulated them by CD3 crosslinking. The relatively low frequency of memory CD8$^+$ T cells generated from physiologic naive cell input numbers and low protein yield precluded analysis of ZAP-70 phosphorylation. However, as seen with effector CD8$^+$ T cells, exposure to inflammation resulted in dramatically increased phosphorylation of ERK1 and ERK2 after TCR ligation (Figures 7A and 7B). This difference in ERK1 and ERK2 phosphorylation was no longer observed when TCR proximal signaling was bypassed with PMA (Figure 7C), demonstrating that exposure to inflammatory cytokines also enhanced proximal TCR signaling in memory CD8$^+$ T cells. Collectively, these data demonstrate that inflammatory

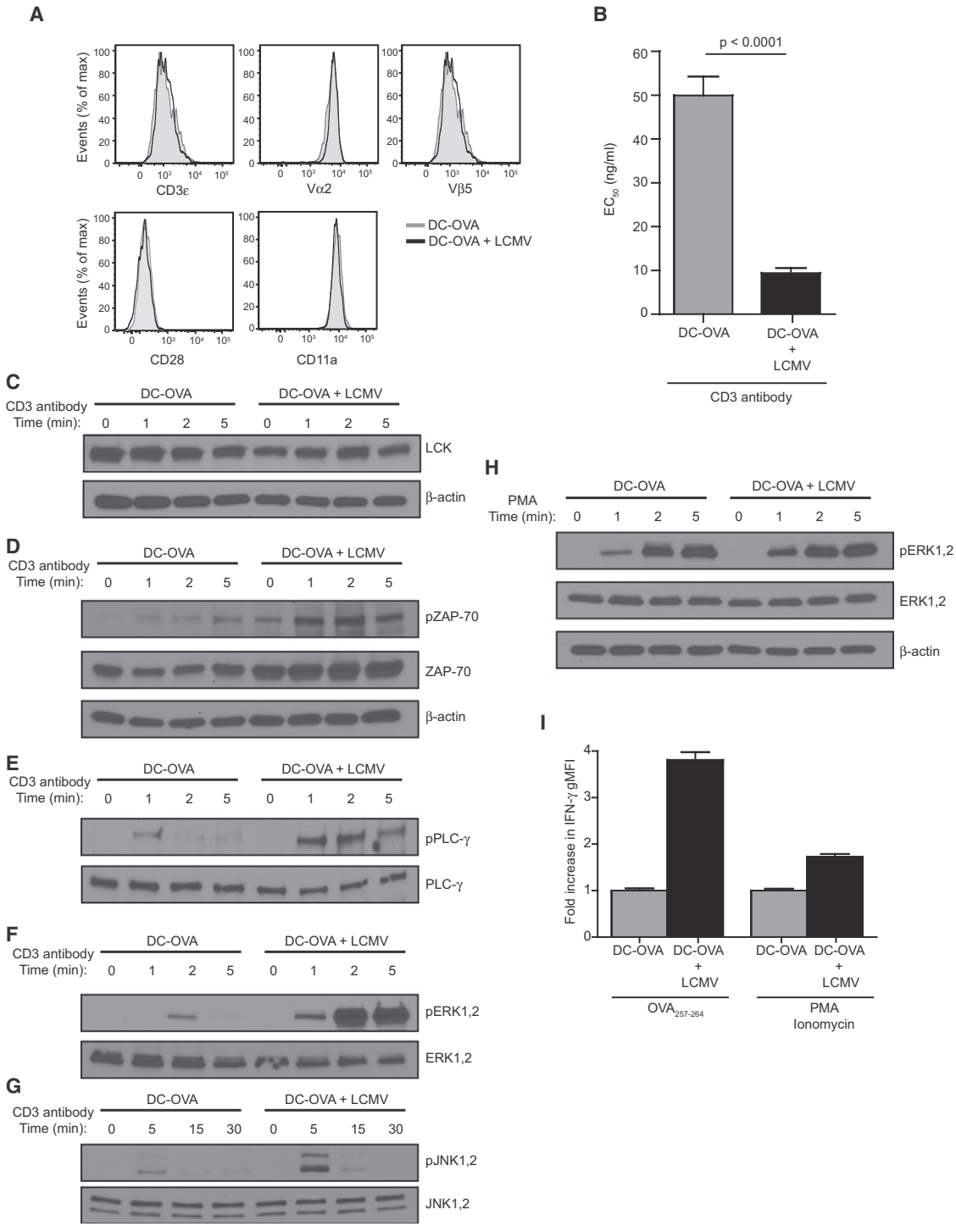

Figure 4. Inflammation Enhances TCR Proximal Signaling of CD8⁺ T Cells

(A) Representative plots of surface expression of the indicated marker at day 5 after immunization with DC-OVA alone (gray filled histograms) or DC-OVA and LCMV (black lines). Data are representative of two independent experiments.

(B) Summary (mean ± SEM) of EC_{50} for IFN-γ production by OT-I $CD8^+$ T cells after ex vivo stimulation with titrated concentrations of plate-bound CD3 antibody. Data are cumulative from two independent experiments with six mice per group and were analyzed with a two-tailed, unpaired Student's t test.

(C–G) Immunoblot analysis of cell lysates from OT-I $CD8^+$ T cells for expression of the protein or phosphorylated protein indicated on day 5 after DC-OVA immunization with or without LCMV coinfection. Cells were stimulated by CD3-antibody crosslinking as indicated. Equivalent amounts of total protein were loaded in each lane. Samples were pooled from at least five to ten mice.

(H) The same as (C) but for cells stimulated with PMA.

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cytokines tune antigen sensitivity by regulating TCR signaling of both effector and memory CD8$^+$ T cells.

DISCUSSION

Generation of highly sensitive CD8$^+$ T cell clones is important for the clearance of pathogen-infected cells, which can express low amounts of antigen (Alexander-Miller, 2005; Walker et al., 2010). Herein, we have demonstrated that inflammatory cytokines directly regulate the antigen sensitivity of CD8$^+$ T cells independently of clonal selection. Our data have highlighted that the antigen sensitivity of CD8$^+$ T cells is not hardwired but rather is dynamically regulated in a pathogen-specific manner. Further, we have demonstrated that exposure to inflammatory cytokines enhances TCR signaling of early effector and preexisting memory CD8$^+$ T cells to control antigen sensitivity. Dynamic tuning of TCR signaling by pathogen-specific inflammatory cytokines most likely represents an important regulatory mechanism for the induction of optimal CD8$^+$ T cell immunity.

Inflammatory cytokines provide a third signal that is required for optimal accumulation of effector CD8$^+$ T cells (Curtsinger and Mescher, 2010; Curtsinger et al., 1999; Curtsinger et al., 2005) and for sustaining effector differentiation (Badovinac et al., 2005; Joshi et al., 2007; Joshi and Kaech, 2008; Pham et al., 2009). Here, we have extended the role of these inflammatory cytokines by demonstrating their capacity to modulate the antigen sensitivity of individual CD8$^+$ T cell clones. We have shown that the magnitude of increase in antigen sensitivity of the same CD8$^+$ T cell clone varies depending on the inflammatory milieu induced by the pathogen used for infection. Thus, the precise pattern of inflammatory cytokines induced by infection can help tune antigen sensitivity in order to achieve optimal CD8$^+$ T cell responses.

The rapid induction of peak antigen sensitivity by inflammatory cytokines might be of critical importance for the efficient elimination of pathogens. CD8$^+$ T cells with high antigen sensitivity are of particular importance for the clearance of viral pathogens. For example, patients that clear hepatitis C virus infection contain CD8$^+$ T cells with higher antigen sensitivity than do CD8$^+$ T cells harvested from chronically infected patients (Yerly et al., 2008). Here, we have demonstrated that induction of inflammation with a viral pathogen, LCMV, results in a nearly 10-fold increase in the antigen sensitivity of OT-I CD8$^+$ T cells and allows for more efficient in vivo killing of targets coated with a low density of antigen. Such increases in antigen sensitivity could allow antigen-specific CD8$^+$ T cells to carry out effector function more efficiently during the course of infection by targeting cells when they are expressing lower amounts of antigen. This could be of particular importance because many viral pathogens are known to interfere with major-histocompatibility-complex class I trafficking and expression, and this interference would lead to lower amounts of cognate antigen expressed on infected cells (Donaldson and Williams, 2009; Griffin et al., 2010; Jackson et al., 2011). It should be noted that, although exposure of responding CD8$^+$ T cells to inflammation enhanced antigen sensitivity, this was balanced by the fact that these cells were slower to acquire memory characteristics, including the capacity for robust secondary expansion, than were cells primed in a low-inflammatory environment (Badovinac et al., 2005; Haring et al., 2006). As such, although enhanced antigen sensitivity might represent a significant advantage for short-term protection, the consequences of these changes for long-term protection and memory will require further study and must be considered during therapy design or the development of vaccine strategies.

It has previously been established that tissue-specific infection results in nonspecific recruitment of memory T cells to inflamed sites (Ely et al., 2003; Kohlmeier et al., 2008). We have demonstrated that exposure to inflammatory cytokines also dynamically enhances the antigen sensitivity of memory CD8$^+$ T cells in the absence of antigen re-encounter in both the spleen and peripheral tissue. This suggests that infection enhances the antigen sensitivity of memory CD8$^+$ T cells regardless of their antigen specificity, thus preparing these cells for rapid deployment of effector functions should they encounter cells expressing low amounts of cognate antigen. Rapid enhancement in antigen sensitivity might in part explain why memory T cells respond to an invading pathogen by producing effector cytokines within hours of infection (Whitmire et al., 2008). Bystander activation of memory CD8$^+$ T cells also occurs during acute infection in humans, suggesting that this represents a conserved regulatory mechanism for optimal memory CD8$^+$ T cell function (Odumade et al., 2012).

A previous study suggested that antigen exposure results in hardwired TCR-signaling-pathway changes that increase antigen sensitivity in a temporal fashion (Slifka and Whitton, 2001). This conclusion was drawn during the evaluation of the CD8$^+$ T cell response to a single pathogen, and although some changes in TCR signaling are likely to occur solely as a result of antigen exposure, our results show that TCR-signaling capacity can be dynamically tuned by exposure to pathogen-specific inflammatory cytokines in both effector and memory CD8$^+$ T cells. Indeed, we observed that inflammatory cytokines rapidly and transiently enhance the antigen sensitivity of effector CD8$^+$ T cells. Similarly, we have shown that the antigen sensitivity of a monoclonal population of memory CD8$^+$ T cells can be transiently increased after exposure to inflammation. This demonstrates that the antigen sensitivity of circulating memory T cells that do not re-encounter their cognate antigen is blunted as inflammatory stimuli wane. As such, the modulation of the antigen sensitivity of memory cells most likely represents an important regulatory mechanism aimed at minimizing the risks of immunopathology or autoimmunity.

One major question that remains is how inflammatory cytokines modulate the signaling capacity of responding CD8$^+$ T cells. Inflammatory cytokines induce the transcription of a

(I) Summary fold increase in gMFI of IFN-γ expression after stimulation with OVA peptide (10 nM) or PMA and ionomycin. Data are presented as the fold increase compared to the mean gMFI of IFN-γ production by OT-I CD8$^+$ transgenic T cells from mice immunized with DC-OVA only. Data are cumulative of two independent experiments with at least six mice per group.
See also Figure S3.

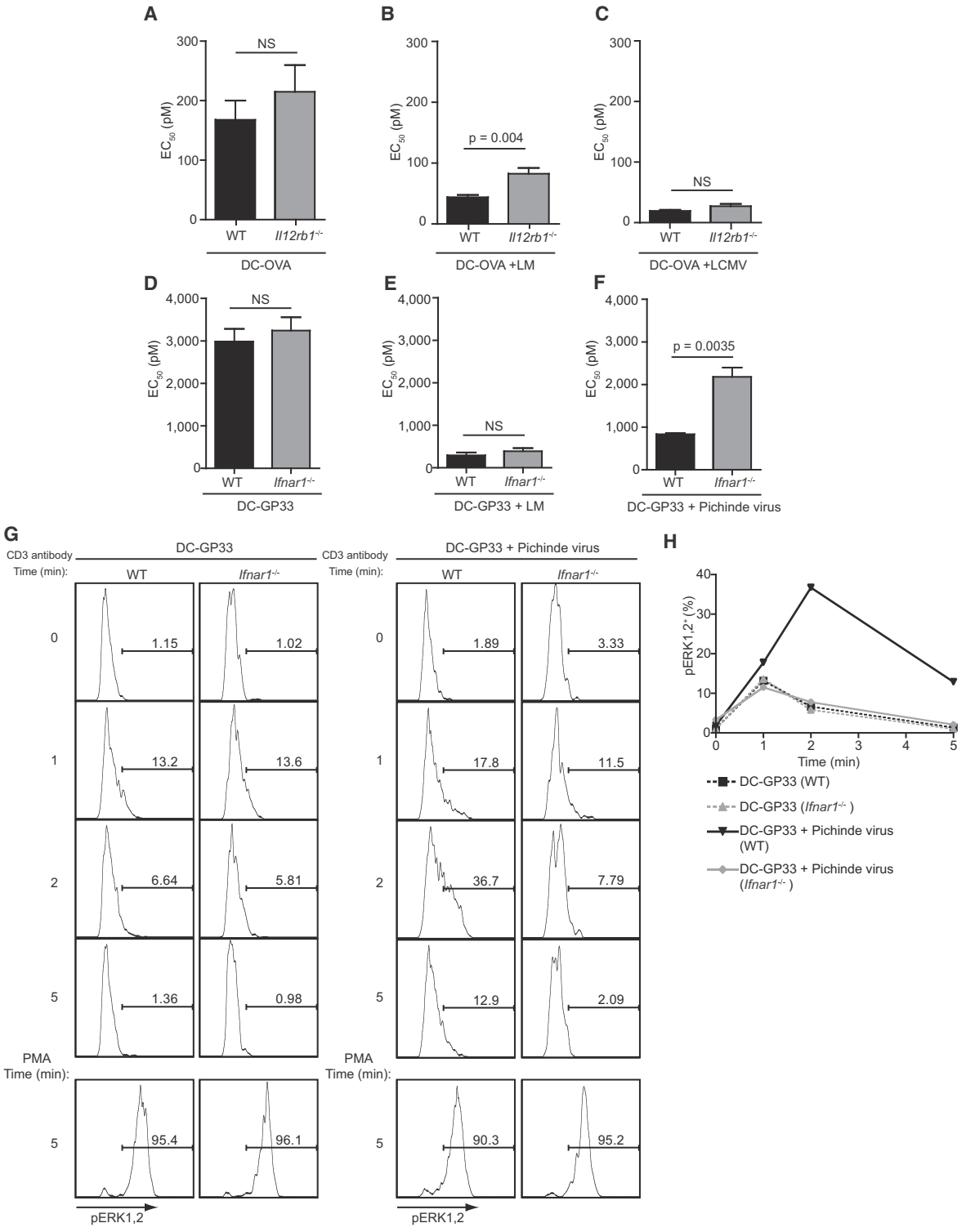

Figure 5. Inflammatory Cytokines Regulate the Antigen Sensitivity of CD8$^+$ T Cells

(A) Summary (mean ± SEM) of EC_{50} for IFN-γ production by congenically marked WT or *Il12rb1*$^{-/-}$ OT-I CD8$^+$ T cells adoptively transferred into the same WT hosts at day 5 after immunization with DC-OVA alone. Data are cumulative (mean ± SEM) from two independent experiments with at least six mice per group. (B and C) The same as (A) but for mice immunized with DC-OVA and coinfected with (B) LM or (C) LCMV.

(D–F) Same as (A)–(C), respectively, but for WT or *Ifnar1*$^{-/-}$ P14 CD8$^+$ T cells adoptively transferred into WT hosts immunized with (D) DC-GP33 alone or infected on day 3 after immunization with (E) LM or (F) PV. Data are from three mice per group (mean ± SEM), are representative of two independent experiments, and were analyzed with a two-tailed, unpaired Student's t test.

(legend continued on next page)

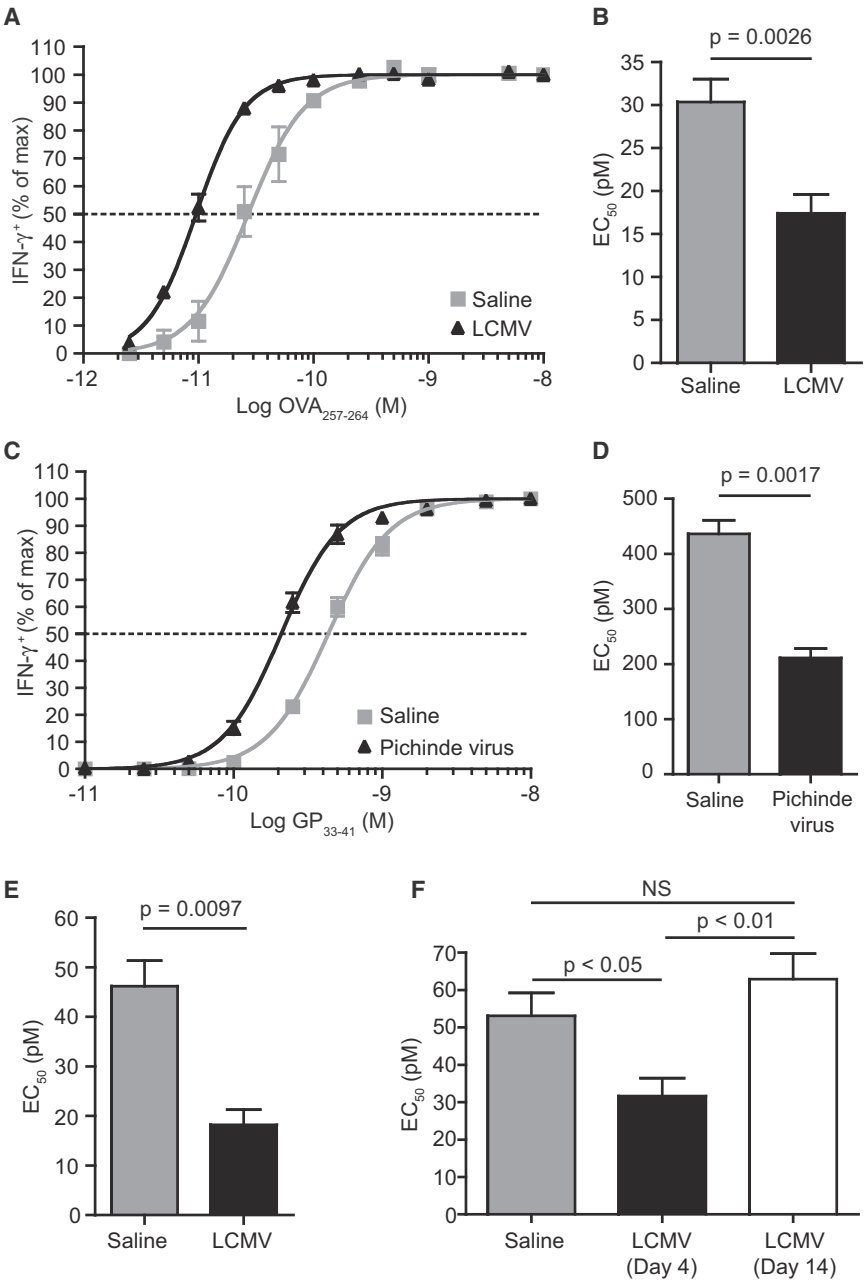

Figure 6. Inflammatory Cytokines Enhance the Antigen Sensitivity of Memory CD8$^+$ T Cells

(A) The percentage of IFN-γ^+ memory OT-I CD8$^+$ T cells at day 4 after infection with LCMV (black triangles) or mock infection with saline (gray squares) was determined after ex vivo stimulation with titrated concentrations of OVA peptide. Data are normalized as in Figure 1 except that peptide saturation was 10 nM.

(B) Summary (mean ± SEM) of EC_{50} for IFN-γ production by memory OT-I CD8$^+$ T cells.

(C and D) Same as (A) and (B), respectively, but for memory P14 CD8$^+$ T cells after ex vivo stimulation with GP_{33-41} peptide on day 4 after infection with PV (black triangles) or mock infection with saline (gray squares).

(E) Same as (B) but for memory OT-I CD8$^+$ T cells isolated from the lung at day 3 after intranasal infection with LCMV (black triangles) or mock infection with saline (gray squares). Saturating peptide = 10 nM.

(F) Same as (B) but for memory OT-I T cells on day 4 (black bars) or day 14 (white bars) after infection with LCMV or mock infection with saline (gray bars).

Data in (A) and (C)–(E) are from three mice per group and are representative of at least two independent experiments. Data in (B) and (F) are cumulative from at least two independent experiments with at least six mice per group. Data in (B), (D), and (E) were analyzed with a two-tailed, unpaired Student's t test. Data in (F) were analyzed by one-way ANOVA with Tukey's post-test of multiple comparisons. See also Figure S4.

variety of genes, including those encoding transcription factors, and could lead to changes in the expression of key signaling components (Agarwal et al., 2009; Joshi et al., 2007; Pipkin et al., 2010). Slifka and Whitton (2001) reported a correlation between the amount of total LCK expression and the amount of IFN-γ per CD8$^+$ T cells. They suggested that the total expression of this signaling molecule might regulate antigen sensitivity of CD8$^+$ T cells. However, we have demonstrated that exposure to inflammatory cytokines directly enhances the signaling capacity of early effector CD8$^+$ T cells without increasing the total expression of either LCK or ZAP-70. This suggests that cytokine signaling to CD8$^+$ T cells enhances the activity of signal-transducing molecules involved in the TCR-signaling pathway. To this effect, a recent study has demonstrated that antigen stimulation of CD8$^+$ T cells results in an increase in oligomeric TCR complexes (Kumar et al., 2011). A contribution of inflammatory cytokines to the reorganization of TCR complexes could explain the increased sensitivity of memory T cells to antigen stimulation. Alternatively, exposure to inflammatory cytokines might reduce the expression or activity of negative TCR-signaling regulators, such as phosphatases. Resolving these issues will require further studies.

(G) Representative flow-cytometry plots of pERK1 and pERK2 from WT or *Ifnar1*$^{-/-}$ P14 CD8$^+$ T cells on day 5 after immunization with DC-GP33 with or without PV infection on day 3. Cells were stimulated with either CD3-antibody crosslinking or PMA for the indicated times. Data are from three pooled mice per group and are representative of two independent experiments.

(H) Summary data of the percentage of pERK1 and pERK2 from WT or *Ifnar1*$^{-/-}$ P14 CD8$^+$ T cells after activation by CD3-antibody crosslinking. Data are from three pooled mice per group and are representative of two independent experiments.

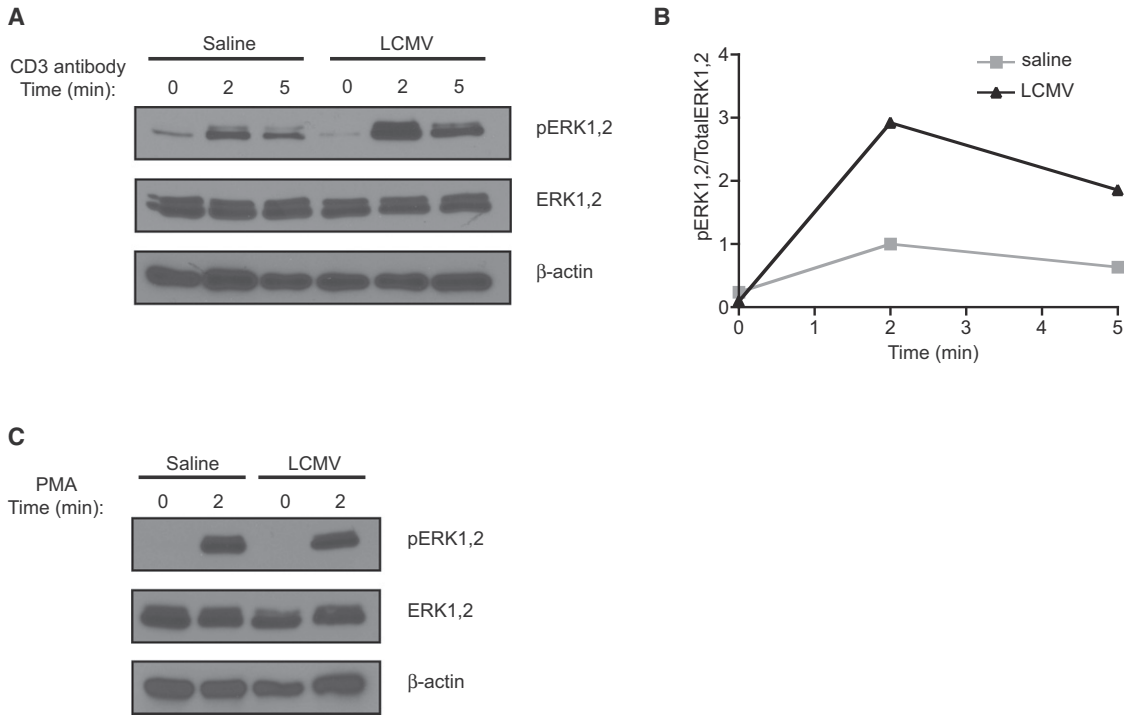

Figure 7. Inflammatory Cytokines Enhance TCR Signaling of Memory CD8$^+$ T Cells

(A) Immunoblot analysis of cell lysates from memory OT-I CD8$^+$ T cells on day 4 after infection with LCMV or mock infection with saline. Cells were stimulated by CD3-antibody crosslinking as indicated. Equivalent amounts of protein were loaded in each lane. Samples were pooled from five to ten mice and are representative of two independent experiments.

(B) The relative signal intensity of pERK1 and pERK2 is divided by the relative signal intensity of total ERK1 and ERK2. Data are normalized to the maximal activation observed in memory CD8$^+$ T cells from mock-infected mice. Samples were pooled from five to ten mice.

(C) The same as (A) but for cells stimulated with PMA.

In conclusion, TCR-signaling capacity can be directly regulated by inflammatory cytokines, resulting in T-cell-intrinsic increases in antigen sensitivity and in vivo cytolytic capacity. Our study demonstrates that the antigen sensitivity of individual CD8$^+$ T cell clones is not hardwired after infection but rather is a dynamic process dictated by the specific inflammatory milieu induced by an invading pathogen. This most likely allows the rapid generation of optimal effector and memory CD8$^+$ T cell responses while minimizing the risk of immunopathology.

EXPERIMENTAL PROCEDURES

Mice, Pathogens, and Dendritic Cells

C57BL/6 mice were purchased from the National Cancer Institute (Frederick, MD, USA). Mice with TCR tg OT-I cells and P14 cells were previously described (Hogquist et al., 1994; Pircher et al., 1987). *Il12rb1*$^{-/-}$ OT-I cells were provided by Dr. Mescher (University of Minnesota), and *Ifnar1*$^{-/-}$ P14 cells were provided by Dr. Murali-Krishna Kaja (Emory University). Infected mice were housed at the appropriate biosafety level. The University of Iowa Animal Care and Use Committee approved animal experiments. An attenuated *act-A*-deficient strain of LM (DPL1942) (Brundage et al., 1993) was grown and injected intravenously (i.v.) (1×10^6 colony-forming units [cfu]) as described (Harty and Bevan, 1995). LCMV was propagated as described (Slifka and Whitton, 2001) and injected intraperitoneally (i.p.) (2×10^5 plaque-forming units [pfu]). PV was propagated as described (Varga et al., 2001) and injected i.p. (1×10^6 pfu). LPS-matured OVA-peptide-coated DCs were prepared as previously described (Schmidt et al., 2008) and injected i.v. (5×10^5).

Adoptive Transfers and Generation of Memory Cells

Naive TCR tg CD8$^+$ T cells (1×10^3 OT-I Thy1.1/1.1 or Thy1.1/1.2 cells) were injected i.v. into naive Thy1.2 recipients. Mice were immunized 24 hr later with DC-OVA with or without infection with LM or LCMV. *Il12rb1*$^{-/-}$ Thy1.2/1.2 OT-I cells were mixed 1:1 with WT Thy1.1/1.1 OT-I cells (1×10^3 each) and injected i.v. into naive CD45.1 recipients and immunized as indicated. Thy1.1/1.1 *Ifnar1*$^{-/-}$ P14 cells were mixed 1:1 with WT Thy1.1/1.2 P14 cells (5×10^3 each), injected i.v. into naive Thy1.2/1.2 recipients, immunized with DC-GP33 on day 0, and subsequently infected with LM or PV on day 3 postimmunization. For generation of primary memory cells, naive Thy1.1 OT-I CD8$^+$ T cells (5×10^2) or P14 CD8$^+$ T cells (1×10^4) were injected i.v. into naive Thy1.2 WT recipients infected with either *actA*$^{-/-}$ LM OVA (5×10^6 cfu) or LCMV (2×10^5 pfu). Mice were rested at least 50 days before use.

Ex Vivo Cytokine and Degranulation Analyses

Ex vivo cytokine detection and degranulation assays were performed as previously described (Butler et al., 2012; Nolz and Harty, 2011) and as detailed in the Supplemental Experimental Procedures. Where indicated, congenically marked cells from DC-OVA only and coinfected groups were mixed and stimulated in the same well. Titration curves were fitted by nonlinear regression.

In Vivo Cytolytic Assay

Assay was performed as previously described (Schmidt et al., 2011). In brief, splenocytes were left untreated or coated with OVA peptide (0.05 or 0.025 nM) for 1 hr at 37°C. Splenocytes were labeled with 0.8 μM carboxyfluorescein succinimidyl ester (CFSE) (no peptide), 0.02 μM CFSE (0.05 nM OVA), 2.5 μM CellTracker Violet (no peptide), or 0.25 μM (0.025 nM OVA). Labeled cells (5×10^6 each, 2×10^7 total cells) were transferred i.v. into recipient mice. Killing was measured in the spleen at 4 hr after transfer. The

killing percentage was calculated as 100 − (100 × [(% CFSE^{hi} / % CFSE^{lo}) / (% CFSE^{hi} in naive mice / % CFSE^{lo} in naive mice)]) and is presented as the normalized percentage of killing per 1 × 10^6 OT-I CD8$^+$ T cells in recipient mice.

TCR Signaling and Immunoblot Analysis

Naive Thy1.1 OT-I cells were injected i.v. (1 × 10^4) into naive Thy1.2 recipients, which were immunized 24 hr later as indicated. On day 5, spleens were harvested, stained with PE-anti-Thy1.1 antibody (Clone OX-7, BD PharMingen), and purified with PE-antibody magnetic beads according to standard AutoMacs protocols. Memory OT-I CD8$^+$ T cells were purified on day 4 after LCMV infection or mock infection with saline. A total of 1 × 10^6 to 5 × 10^6 cells were incubated on ice for 30 min with 10 μg/ml biotinylated CD3 antibody (clone 145-2C11, eBioscience) and crosslinked with streptavidin or stimulated with 200 ng/ml PMA for the indicated times at 37°C. Cells were washed with cold PBS and lysed in NP40 buffer (20 mM HEPES [pH 7.9], 100 mM NaCl, 5 mM EDTA, 0.5 mM CaCl, 1% Nonidet P-40, 1 mM PMSF, 10 μg/ml leupeptin, 5 μg/ml aprotinin, and 1 mM Na_3VO_4). Five to ten micrograms of protein was resolved by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies as indicated. Antibodies were detected with goat anti-rabbit conjugated to horseradish peroxidase (Santa Cruz) and Supersignal (Thermo Scientific). Images were quantified with ImageJ software. Total-protein quantification is presented as the ratio of the signal intensity of the protein of interest to the signal intensity of β-actin and is normalized to the average ratio for DC-OVA samples. Phosphorylation quantification is presented as the ratio of the signal intensity of the phosphorylated protein of interest to the signal intensity of total protein and is normalized to the maximal phosphorylation in DC-OVA samples. Alternatively, ERK1 and ERK2 phosphorylation was measured by flow cytometry. In brief, congenically marked WT or *Ifnar1*$^{-/-}$ P14 CD8$^+$ T cells (2.5 × 10^4) were adoptively transferred to naive recipients. Mice were immunized with DC-GP33 with or without infection with PV on day 3 postimmunization. Splenocytes were isolated on day 5 postimmunization and stimulated with 10 μg/ml biotinylated CD3 antibody (clone 145-2C11, eBioscience) and crosslinked with streptavidin for the indicated times at 37°C. Cells were fixed with BD cytofix (BD Bioscience), permeabilized with BD Phosflow Perm Buffer III, and stained with an antibody specific to pERK1 and pERK2 (T202 and Y204, respectively). Samples were analyzed with a BDLSRFortessa flow cytometer (BD Bioscience) and flowjo software (Tree Star).

Statistical Analyses

Data were analyzed with GraphPad Prism4 software. Specific tests for determining statistical significance are indicated in the figure legends.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2012.09.017.

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