Regulation of Homeostatic Chemokine Expression and Cell Trafficking During Immune Responses

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Science **317**, 670 (2007);
DOI: 10.1126/science.1144830

whereas extravasated Gr1$^-$ gfp^{high} monocytes turned on—at 2 and 8 hours—genes involved in tissue remodeling (*22*), including arginase1, Fizz1, Mgl2, and mannose receptor (MR) (Fig. 4E and fig. S9).

Indeed, the study of the balance of transcription factors that specifies the alternative macrophage or dendritic cell fate of monocytes (*23*) indicated that extravasated Gr1$^-$ gfp^{high} monocytes initiated a typical macrophage differentiation program, characterized by up-regulation of cMaf and MafB but not RelB and Pu.1 (sfpi1) (Fig. 4G and figs. S6 and S7). In contrast, the conventional Gr1$^+$ gfp^{low} monocytes initiated a DC differentiation program, as described previously (*1*, *6*), by up-regulating RelB and Pu.1 but not cMaf and MafB (Fig. 4G and fig. S6). Analysis of the expression of a larger panel of genes differentially regulated in monocyte-derived macrophages and DC supported this conclusion (fig. S8).

These findings demonstrate a new mechanism of leukocyte crawling on endothelial cells and a new role for LFA-1. The present data also assign a function to gfp^{high} Gr1$^-$ monocytes. Patrolling of blood vessels by these resident monocytes allow rapid tissue invasion by monocytes in case of damage and infection, followed by the initiation of an innate immune response and their differentiation into macrophages. This is in contrast to the role of Gr1$^+$ monocytes, which reach the inflammatory site later and give rise to inflammatory DCs (*1*, *6*). This reveals an unsuspected dichotomy of the differentiation potential and functions of blood monocyte subsets during infection. The extravasation of patrolling Gr1$^-$ gfp^{high} monocytes is likely to be dependent on a yet-unidentified signal(s) from damaged tissue and/or endothelium. Interestingly, the existence of a pool of "marginated" monocytes expressing CD16$^+$ has been proposed in humans (*24*) and may correspond, at least in part, to the patrolling behavior that we describe here, suggesting a similar in vivo function of mouse resident CX$_3$CR1^{high} (gfp^{high}) Gr1^{low} monocytes and human CX$_3$CR1^{high} CD16$^+$ CD14^{low} monocytes. Monocytes are abundant in arthritis and atherosclerotic lesions (*25*, *26*), and CX$_3$CR1, TNF-α, and LFA-1 have been implicated in the pathogenesis of these inflammatory diseases (*27*, *28*); thus, patrolling monocytes may contribute to their pathogenesis and may represent a target for treatment.

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29. We thank M. Sieweke (CIML, Marseille, France) and N. Hogg (Cancer Research UK, London, UK) for expert advice and the gift of reagents. We are also grateful to J. L. Casanova, P. Revy, B. Lucas, and S. Amigorena for expert advice and critical reading of the manuscript; C. Grolleau (Roper Scientific, Paris, France) for expert advice in the use of the Metamorph Software; Jennifer Wong for help in setting up the imaging of mesenteric vessels; and all the members of INSERM U838 for discussion and support. C.A. was a fellow of the Fondation de France. This work was supported by grants (to F.G.) from the Fondation Schlumberger, the Agence Nationale de la Recherche (grant IRAP2005), the City of Paris, the Fondation de France, the Fondation pour la Recherche Médicale, and a European Young Investigator award.

Supporting Online Material

www.sciencemag.org/cgi/content/full/317/5838/666/DC1
Materials and Methods
Figs. S1 to S8
Table S1
References
Movies S1 to S7

22 March 2007; accepted 29 June 2007
10.1126/science.1142883

Regulation of Homeostatic Chemokine Expression and Cell Trafficking During Immune Responses

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The chemokines CCL21 and CXCL13 are immune factors that dictate homing and motility of lymphocytes and dendritic cells in lymphoid tissues. However, the means by which these chemokines are regulated and how they influence cell trafficking during immune responses remain unclear. We show that CCL21 and CXCL13 are transiently down-regulated within lymphoid tissues during immune responses by a mechanism controlled by the cytokine interferon-γ. This modulation was found to alter the localization of lymphocytes and dendritic cells within responding lymphoid tissues. As a consequence, priming of T cell responses to a second distinct pathogen after chemokine modulation became impaired. We propose that this transient chemokine modulation may help orchestrate local cellularity, thus minimizing competition for space and resources in activated lymphoid tissues.

A cardinal property of cells of the immune system is mobility, allowing them to navigate the body and combat invading pathogens. This is modulated by a complex array of chemokines and their receptors, which provide the molecular signals to direct cells to where they are required (*1*). Many chemokines are up-regulated in cells and tissues in response to inflammatory stimuli such as infection (*2*). In contrast, the lymphoid chemokines CCL21, CCL19, and CXCL13 are constitutively expressed in restricted areas for steady-state attraction of cells (*3*). CCL21 expression by fibroblastic reticular cells (FRCs) of the T cell zones in the spleen and lymph nodes (LN) facilitates effective interaction between dendritic cells (DCs) and T cells, whereas CXCL13 expression on follicular dendritic cells guides B cells and follicular T helper (T_H) cells into B cell zones (*4*, *5*). The critical role of these homeostatic chemokines in attracting cells into lymphoid organs and in initiating antigen-specific responses is well established (*3*, *6*). However, less is known about the impact that acute immune responses have on the expression of homeostatic chemokines and how this affects lymphocyte trafficking within lymphoid tissues.

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To address these questions, we examined expression of CCL21 and CXCL13 in the spleen by immunofluorescence after infection of mice with lymphocytic choriomeningitis virus (LCMV) (*7*). In contrast to the up-regulation of chemokines observed in peripheral tissues during inflammation (*8*, *9*), we found reduced expression of these chemokines in the spleen after LCMV infection (Fig. 1A). To examine this down-regulation in more detail, we assayed spleen homogenates for CCL21 and CXCL13 protein by enzyme-linked immunosorbent assay (ELISA). Down-regulation of the lymphoid chemokines was observed in the spleen (Fig. 1B) and also in peripheral LN (Fig. 1C and fig. S1). CCL21 down-regulation began within 3 days of infection, reaching levels 20- to 80-fold less than that detected before infection within 8 days (Fig. 1B). Importantly, modulation was transient, and we observed relatively normal CCL21 expression within 2 weeks after LCMV infection (Fig. 1B). The down-modulation of CXCL13 was also transient but with slightly delayed kinetics: levels remained low at 2 weeks after infection and returned to normal within 5 weeks (Fig. 1B). This transient modulation of the homeostatic chemokines correlated with the generation of virus-specific T and B cell responses after infection.

To determine whether regulation of lymphoid chemokine expression after infection occurred at the transcriptional level, we quantified CCL21 and CXCL13 mRNA by real-time reverse transcription polymerase chain reaction (RT-PCR). About 100-fold less CCL21 mRNA and 10-fold less CXCL13 mRNA was detectable in whole spleens, as well as in isolated cells of the

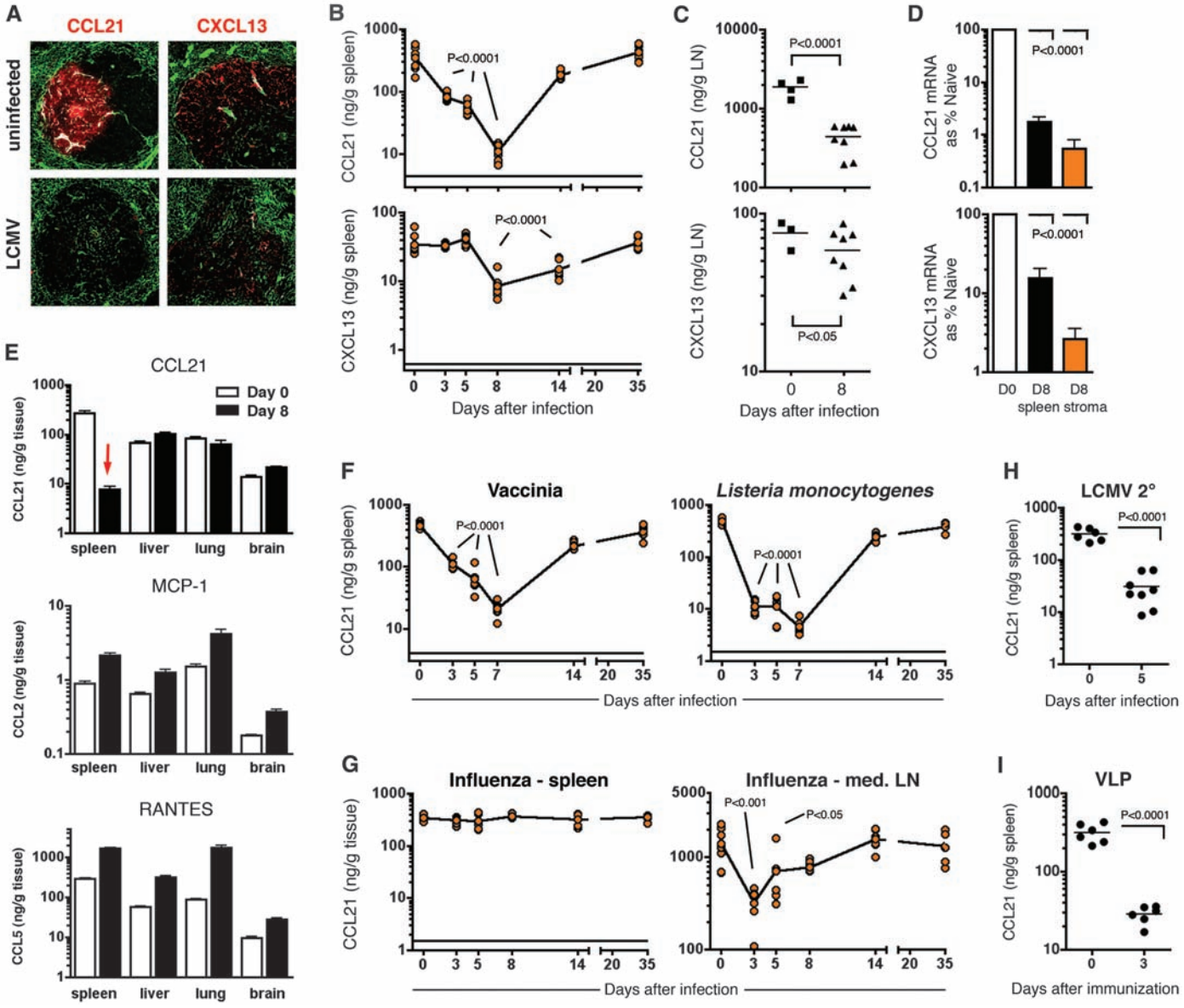

Fig. 1. Transient down-regulation of the lymphoid chemokines occurs during immune responses. (**A**) Spleens from uninfected mice (day 0), or 8 days after LCMV infection, stained for ER-TR7 (green) to detect FRC and anti-CCL21 or anti-CXCL13 (red). White regions indicate co-localization; objective magnification, 20×. (**B** and **C**) Lymphoid chemokine expression in the spleen and inguinal LN after LCMV infection, quantified by ELISA in tissue homogenates. (**D**) Quantitative RT-PCR analysis of chemokine expression in whole spleen or the stromal compartment 0 or 8 days after LCMV infection. Error bars indicate SEM. (**E**) Down-regulation of CCL21 was restricted to lymphoid organs (arrow). CCL21, CCL2 (MCP-1), and CCL5 (RANTES) expression in tissues 0 and 8 days post-LCMV infection. (**F** to **I**) Chemokine modulation during viral and bacterial infections. (F) CCL21 expression in the spleen after systemic VV infection or LM infection, (G) in the draining mediastinal LN and the spleen after intranasal influenza virus infection, (H) in the spleens of LCMV immune mice after secondary infection with LCMV, and (I) in the spleen after immunization with VLPs. *P* values were calculated by *t* test, relative to uninfected mice.

splenic stromal compartment, after LCMV infection (Fig. 1D). Down-regulation of CCL19 protein as well as mRNA was also observed in the lymphoid organs after LCMV infection (fig. S2).

Infection and inflammation can induce up-regulation of various lymphocyte-attracting chemokines in both lymphoid and nonlymphoid tissues (*10*). Interestingly, we found that down-regulation of CCL21 was restricted to lymphoid tissues (Fig. 1E). Yet, up-regulation of the inflammatory chemokines CCL2 and CCL5 occurred in both lymphoid and nonlymphoid tissues (Fig. 1E), indicating that down-regulation was specific to the homeostatic chemokines. To determine whether lymphoid chemokine modulation is a specific feature of LCMV infection or it is a more generalized feature of immune responses to pathogens, we examined chemokine levels after infection of mice with vaccinia virus (VV) or *Listeria monocytogenes* (LM). We observed marked down-regulation of CCL21 and CXCL13 in the spleen after infection with these pathogens (Fig. 1F and fig. S3). We also infected mice intranasally with influenza virus. Although down-regulation of lymphoid chemokine expression was not observed in the spleen after influenza infection, CCL21 and CXCL13 expression was modulated in the draining mediastinal LN (Fig. 1G and fig. S3). These data show that transient chemokine down-regulation occurs during both systemic and localized infections and also make the important point that modulation of lymphoid chemokine expression occurs primarily at the site of the antigen-driven immune response.

A recent study described changes in CCL21 expression after mouse cytomegalovirus infection (*11*) and attributed this to virus-induced pathology. However, the modulation of chemokine expression that we found after infection with both viral and bacterial pathogens indicated that this represented a generalized programmed response. We also observed down-regulation of CCL21 during recall responses (Fig. 1H) and after immunization with virus-like particles (VLPs) containing CpG (Fig. 1I). Similar results were obtained after immunization with ovalbumin (OVA) protein plus lipopolysaccharide

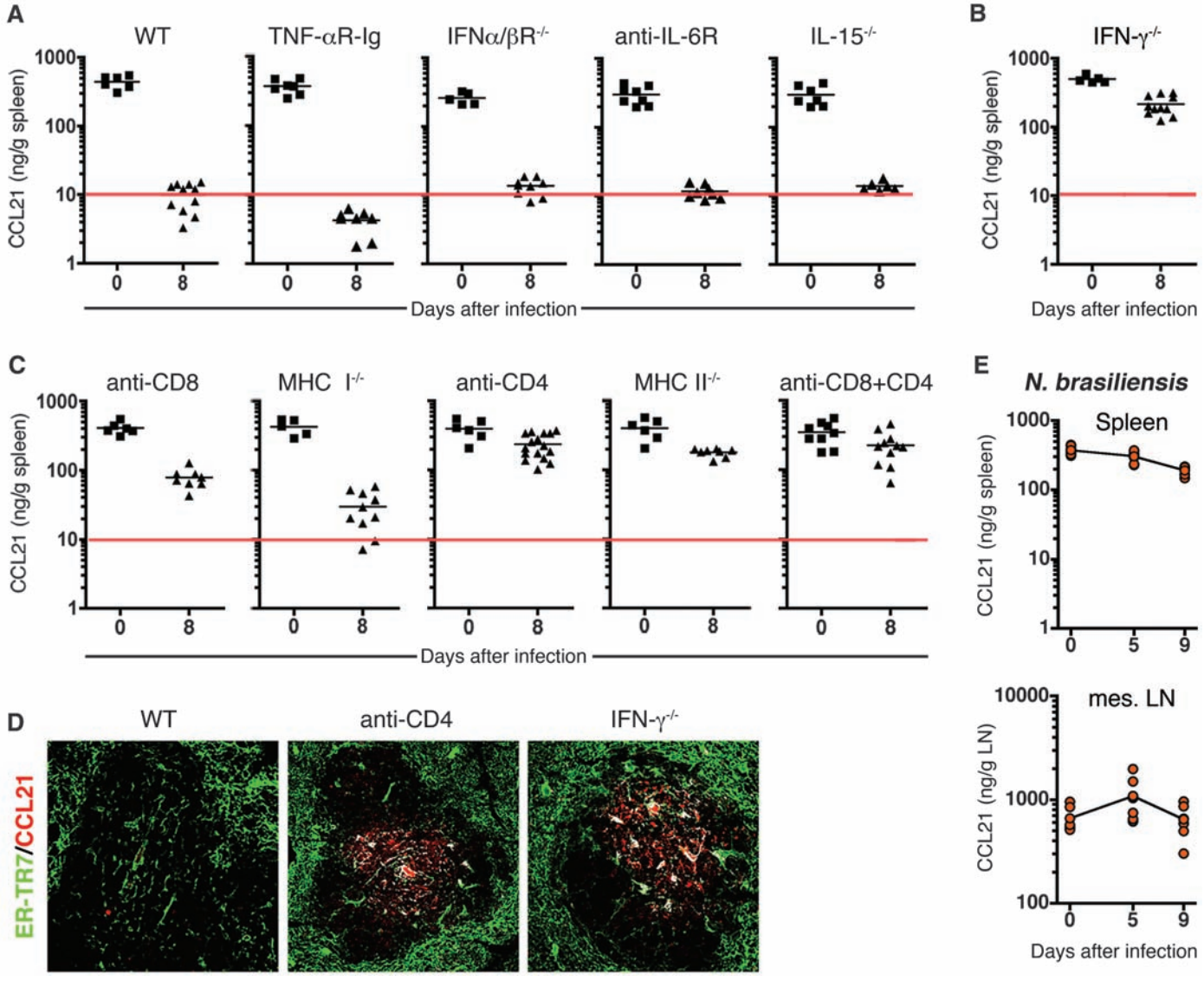

Fig. 2. Role of $CD4^+$ T cells and IFN-γ in lymphoid chemokine down-regulation. (**A**) CCL21 expression after LCMV infection in spleens of wild-type (WT), IFNα/$βR^{-/-}$, or IL-$15^{-/-}$ mice or mice treated with sTNF-αR-Ig or IL-6R antibody. (**B**) CCL21 expression in IFN-$γ^{-/-}$ mice. (**C**) CCL21 expression after LCMV infection in spleens of WT mice depleted of $CD8^+$ T cells (anti-CD8), $CD4^+$ T cells (anti-CD4), or both (anti-CD8+CD4) or in mice deficient in MHC class I or class II molecules. Statistical analysis was performed on day 8 samples, relative to WT values: $P < 0.0001$ for IFN-$γ^{-/-}$, anti-CD8, anti-CD4, anti-CD8+CD4 and MHC-$II^{-/-}$; for MHC $I^{-/-}$, $P = 0.0019$. Horizontal red lines represent mean CCLR21 expression in WT mice 8 days after LCMV infection. (**D**) CCL21 expression in spleens from WT, anti-CD4, and IFN-$γ^{-/-}$ mice 8 days after infection. Objective magnification, 20×. (**E**) CCL21 expression in spleen and mesenteric LN after subcutaneous infection with *N. brasiliensis*.

(LPS) as an adjuvant, suggesting that pathogen replication was not necessary for chemokine modulation. Together, these results suggest that lymphoid chemokine regulation is an integral feature of adaptive immune responses.

Although lymphotoxin β receptor (LTβR) signals are important for lymphoid chemokine expression during organogenesis (*12*), activation or inhibition of the LTβR pathway had minimal effect on CCL21 modulation after infection [Supporting Online Material (SOM) text 1 and fig. S4] (*11*). To understand the mechanism of lymphoid chemokine down-regulation, we determined that many innate cytokines produced after infection, such as tumor necrosis factor α (TNF-α), interferon α and interferon β (IFN-α/β), interleukin-6 (IL-6), and IL-15, were not required for this lymphoid chemokine down-regulation (Fig. 2A and fig. S4). Our data indicate that IL-12 and IL-1 may play a minor role in lymphoid chemokine regulation (fig. S4). Importantly, IFN-γ was required, because lymphoid chemokine down-regulation was only minimally seen after infection of IFN-γ–deficient mice (Fig. 2, B and D).

To ascertain the role of T cells, we infected mice lacking major histocompatibility complex (MHC) class I or II molecules or $CD8^+$ or $CD4^+$ T cells. Minimal down-regulation of chemokine expression occurred in MHC $II^{-/-}$ or CD4-depleted mice, indicating that $CD4^+$ T cells were required (Fig. 2, C and D). To further examine the role of $CD4^+$ T cells and IFN-γ production (T_H1) in chemokine modulation during immune responses, we infected mice with the nematode *Nippostrongylus brasiliensis* or the protozoa *Leishmania major*. These parasitic infections induce strong T_H2 immune responses, whereas other responses we analyzed were T_H1-driven. We observed minimal modulation of CCL21 or CXCL13 in spleen or LN after *N. brasiliensis* infection and after *L. major* infection (Fig. 2E and fig. S5). Thus, we demonstrate two components of type I immune responses ($CD4^+$ T cells and IFN-γ) that are important for lymphoid chemokine modulation during immune responses.

To determine the functional consequences of lymphoid chemokine down-regulation during an ongoing immune response, we examined the migration of naïve carboxyfluorescein diacetate succinimidyl ester (CFSE)–labeled $CD8^+$ T cells in the spleen, after adoptive transfer into normal and infected mice (Fig. 3A). In uninfected mice, the transferred naïve T cells localized predominantly to the T cell zones of the white pulp (WP) (71 ± 19.8%; Fig. 3, B and F). In contrast, in LCMV-infected mice the transferred T cells accumulated in the red pulp (RP), and few were found in the WP (4.4 ± 7.2%). This alteration in naïve lymphocyte localization in the spleen after infection correlated with the degree of CCL21 down-regulation (Fig. 1): Migration into the T cell zone was intact for at least 3 days after infection, whereas an intermediate phenotype was observed 5 days after infection (Fig. 3, B and F). The abrogation of naïve T cell movement into the WP was directly dependent on CCL21 expression, because mice lacking $CD4^+$ T cells or IFN-γ demonstrated normal migration of $CFSE^+$ naïve T cells to the T cell zones (Fig. 3, C and F), indicative of CCL21 expression in these mice. Similar results were obtained with transferred naïve $CD4^+$ T cells (fig. S6). Furthermore, mice infected with LCMV in the footpad demonstrated

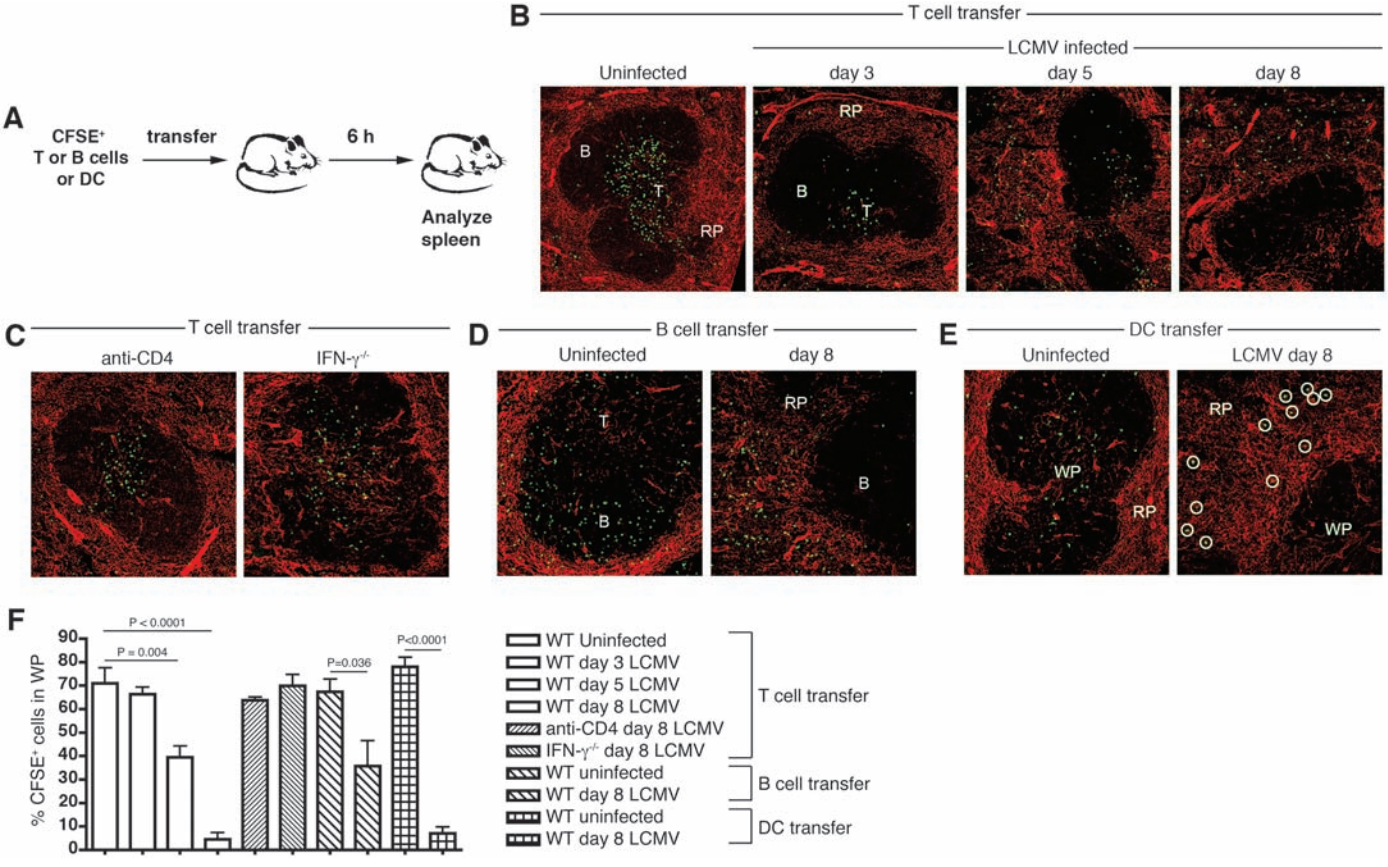

Fig. 3. Altered cell localization after lymphoid chemokine down-regulation. (**A** to **E**) Localization of transferred lymphocytes or DCs in the spleen after infection. (A) Naïve T or B lymphocytes or splenic DCs were purified and labeled with CFSE and transferred into mice, and their localization in the spleen was ascertained 6 hours later. $CFSE^+$ $CD8^+$ T cells were transferred into (B) WT mice 0 to 8 days after LCMV infection, or into (C) CD4-depleted (anti-CD4), or into IFN-$\gamma^{-/-}$ mice 8 days after infection. (D) $CFSE^+$ B cells or (E) $CFSE^+$ DCs were transferred into uninfected or day 8 LCMV-infected mice. Spleen sections were co-stained with ER-TR7 (red) to determine the localization of the $CFSE^+$ (green) cells. Objective magnification, 20×; RP, red pulp; WP, white pulp; B, B cell zone; and T, T cell zone. (**F**) Quantitation of $CFSE^+$ cells in the spleen after transfer. The proportion of $CFSE^+$ cells present in WP regions is shown. Data are representative of 5 to 9 mice per group. Error bars indicate SEM.

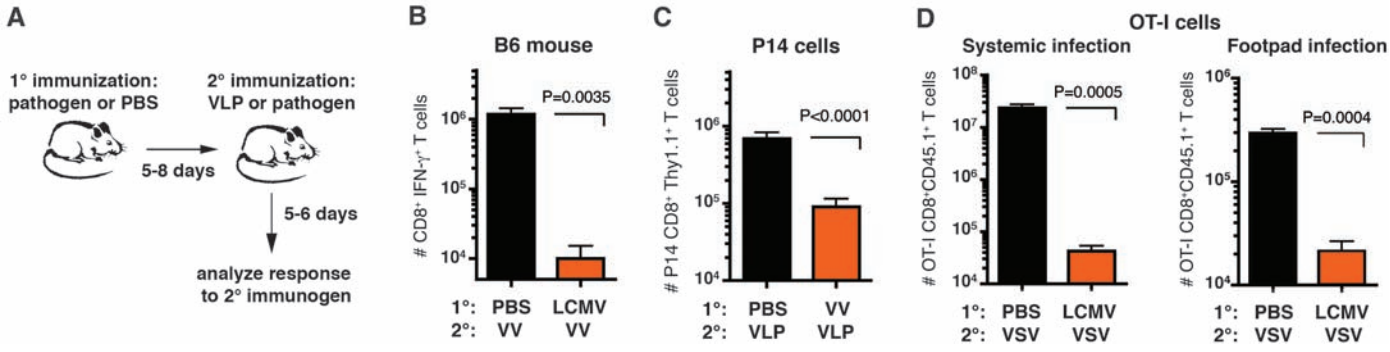

Fig. 4. Impaired priming of naïve CD8$^+$ T cells after lymphoid chemokine modulation. (**A**) Schematic of primary-secondary immunization experiments. Mice were infected with a primary pathogen or given PBS, followed by immunization with VLP or infection with a secondary pathogen after modulation of the lymphoid chemokines (day 5 for VV and day 8 for LCMV). (**B**) Expansion of VV-specific IFN-γ^+ CD8$^+$ T cells is shown in spleens 6 days after VV co-infection of LCMV-infected or PBS-treated mice. (**C**) Expansion of P14 Thy1.1$^+$ CD8$^+$ T cells in the spleen 6 days after VLP immunization of VV-infected mice. (**D**) Expansion of OT-I CD45.1$^+$ CD8$^+$ T cells in the spleen (systemic infection) or popliteal LN (footpad infection) 5 days after VSV-OVA infection of LCMV-infected mice. Representative data from two to three experiments are shown. Error bars indicate SEM.

a significant reduction in naïve T cell entry into the draining popliteal LN after chemokine modulation (fig. S7).

We also observed reduced migration of naïve B cells into B cell zones after infection (Fig. 3, D and F). Yet, cells were observed to home to some B cell zones, potentially reflecting the degree of local CXCL13 regulation. Differential regulation of CCL21 and CXCL13 may allow proper orchestration of T and B cell responses. Lastly, because the migration of activated DC into T cell zones is also dependent upon responsiveness to CCL21 (*13*, *14*), we examined the localization of CFSE-labeled mature DCs in the spleen after infection. DCs migrated into the T cells zones of uninfected mice, yet localized to the RP and also to the marginal zone, after infection (Fig. 3, E and F). Together, these data demonstrate that lymphoid chemokine modulation altered lymphocyte and DC localization within lymphoid T and B cell zones, potentially regulating local cellularity and antigen presentation.

These results suggested that priming of naïve T cells may be compromised after chemokine down-regulation. To examine this, we gave acutely infected mice a second distinct immunization or infection after chemokine modulation and measured responses to the second antigen (Fig. 4). Mice infected with either LCMV or LM displayed markedly reduced responses to secondary VV or LCMV infection, respectively (Fig. 4B and fig. S8). Similar results were observed in VV-infected mice containing LCMV gp_{33-41}-specific P14 transgenic T cells, which were immunized with VLP containing gp_{33-41} peptide after chemokine modulation (Fig. 4C). Lastly, mice containing OVA-specific OT-I transgenic T cells were infected with LCMV systemically or in the footpad and then co-infected with a recombinant vesicular stomatitis virus expressing OVA (VSV-OVA) when the lymphoid chemokines were most reduced (day 8). Expansion of the OT-I T cells was significantly reduced in comparison with that in control phosphate-buffered saline (PBS)–treated mice (Fig. 4D). Thus, altered localization of naïve T cells after down-regulation of the lymphoid chemokines may affect priming against new antigens during an ongoing response. Although other factors, such as the maturation of DCs and inhibition of cross-presentation (*15*), can affect T cell activation during immune responses, lymphoid chemokine modulation may contribute to such transient immunosuppression. Indeed, activation of naïve P14 T cells transferred after LCMV infection was abrogated corresponding with chemokine modulation, despite the presence of antigen-positive DCs in the spleen that were capable of activating T cells in vitro (fig. S9).

The size of an immune response will likely dictate the magnitude of lymphoid chemokine modulation. However, a drawback of this strategy is that it may be difficult to effectively generate an immune response to a second antigen during the period of transient chemokine down-regulation. This may be particularly relevant during acute viral infections and prime-boost vaccine regimes in instances where only a short delay separates immunization doses.

Our results suggest that, by reducing expression of the homeostatic chemokines in responding lymphoid tissues, the adaptive immune response can orchestrate local cellularity and thus competition for space and resources during ongoing immune responses. Reducing local accumulation of T cells and antigen-presenting cells may be beneficial for shutting down the immune response and promoting the generation of memory cells (SOM text 2). Further, it is possible that CCL21 modulation may work in concert with chemokine receptor and S1P1 regulation on effector cells (*16*) to promote exit from the lymphoid tissues. Because CCL21 is also important for interstitial motility of lymphocytes (*17*–*19*), modulation during immune responses may additionally affect the motility of cells within the lymphoid parenchyma. It will be interesting to determine how lymphocyte movement and trafficking through lymphoid tissues is modulated by changes in homeostatic chemokine expression during immune responses.

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20. We thank J. Browning for murine LTβR-Ig, a fusion protein of mouse LTβR and human immunoglobulin G (hIgG). This work was supported by grant AI30048 from the NIH to R.A.; S.M. was funded by a C. J. Martin postdoctoral fellowship from the National Health and Medical Research Council of Australia. M.M. was supported by research awards from the Arthritis Foundation, the Rosalind Russell Medical Research Center for Arthritis at UCSF, and the Sandler Family Supporting Foundation.

Supporting Online Material

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Materials and Methods
SOM Text
Figs. S1 to S9
References

8 May 2007; accepted 3 July 2007
10.1126/science.1144830