JCI The Journal of Clinical Investigation

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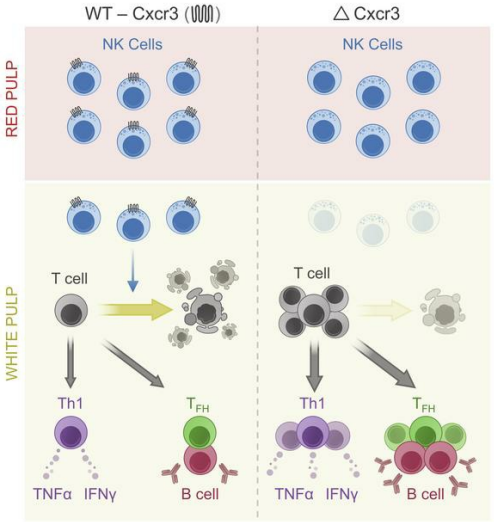
J Clin Invest. 2021. https://doi.org/10.1172/JCI146686.

Concise Communication In-Press Preview Immunology Vaccines

Graphical abstract



Natural killer cell immunosuppressive function requires CXCR3-dependent redistribution within lymphoid tissues.

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The authors have declared that no conflict of interest exists.

Keywords: chemokine, migration, immunoregulation, adenovirus, vaccine, innate lymphoid cell, granzyme

ABSTRACT

Natural killer (NK) cell suppression of T cells is a key determinant of viral pathogenesis and vaccine efficacy. This process involves perforin-dependent elimination of activated CD4 T cells during the first three days of infection. Although this mechanism requires cell-cell contact, NK cells and T cells typically reside in different compartments of lymphoid tissues at steady state. Here, we showed that NK-cell suppression of T cells is associated with transient accumulation of NK cells within T cell-rich sites of the spleen during lymphocytic choriomeningitis virus infection. The chemokine receptor CXCR3 was required for this relocation and suppression of antiviral T cells. Accordingly, NK-cell migration was mediated by type I interferon (IFN)-dependent promotion of CXCR3 ligand expression. In contrast, adenoviral vectors that weakly induced type I IFN and did not stimulate NK-cell inhibition of T cells also did not promote measurable redistribution of NK cells to T-cell zones. Exogenous IFN rescued NK-cell migration during adenoviral vector immunization. Thus, type I IFN and CXCR3 were critical for properly positioning NK cells to constrain antiviral T-cell responses. Development of strategies to curtail migration of NK cells between lymphoid compartments may enhance vaccine-elicited immune responses.

INTRODUCTION

Natural killer (NK) cells are innate lymphocytes with a critical role in immune defense against viruses in both mice and humans (1, 2). In addition to killing virus-infected cells, NK cells regulate antiviral T- and B-cell responses via perforin-dependent elimination of activated CD4 T cells (3, 4). Lymphocytic choriomeningitis virus (LCMV) infection potently triggers immunoregulatory functions of NK cells with significant consequences for viral clearance and immune pathology (3-5). Similar mechanisms constrain humoral immunity after immunization of mice (6, 7). NK-cell suppression of T-cell or antibody responses is also apparent in humans during infections with HIV or hepatitis B virus (HBV) and after administration of yellow fever or HBV vaccines (8-11). An improved understanding of the mechanisms of NK-cell immunosuppressive activity is likely to facilitate development of interventions to enhance vaccine efficacy.

NK-cell suppression of T cells has been reproducibly linked to perforin (3-6, 12), a key component of cell-contact dependent granule-mediated killing (13). Thus, the immunoregulatory activity of NK cells likely involves physical liaisons with T cells in lymphoid tissues. Yet in the absence of inflammation, NK cells are infrequently present at T-cell rich sites, including lymph nodes and the white pulp (WP) of the spleen (14-17). Moreover, NK cells present in human lymph nodes typically express little perforin (18). However, infections with murine cytomegalovirus (MCMV) or LCMV trigger NK-cell accumulation in the WP of spleen (14-16), while infections of humans and non-human primates with HIV or SIV result in localization of NK cells into T/B-rich follicles (19). In this study, we established that positioning of NK cells at T-cell rich sites is essential for NK-cell suppression of antiviral T-cells.

RESULTS AND DISCUSSION

Transient positioning of NK cells in T-cell zones during infection. NK cells eliminate a fraction of virus-specific CD4 T cells via a perforin- and contact-dependent manner in the spleen during the initial three days of LCMV infection in mice (3, 4). Yet at baseline NKp46^{+} NK cells predominately populate the red pulp (RP) regions of the mouse spleen with few NK cells detectable in the T-cell rich WP (**Figure 1A, Figure S1**). Consistent with previous reports (14, 16), infection with LCMV triggered increased proportions (**Figure 1B-C**) and numbers (**Figure 1D**) of NKp46^{+} NK cells in the WP (**Figure 1B, D**) and T-cell zones (**Figure 1C**). This re-localization was apparent at 24 hours and continued to increase until roughly 60% of splenic NK cells localized within the WP by day 3 of infection (**Figure 1A-D**).

To quantify NK-cell localization, we took advantage of differences in vascularity between spleen RP and WP that can be detected using a modification of established intravascular staining methods (20). We intravenously injected an APC-labeled anti-NKp46 antibody and euthanized mice after 3 minutes. Ex vivo staining with anti-NKp46 ubiquitously labels splenic RP NK cells in both infected and uninfected mice (**Figure S2A-B**). Consistent with restricted labeling of NK cells in poorly vascularized sites by intravenous anit-NKp46 antibody (iv-NKp46), NK cells in the WP were shielded from intravascular staining but readily labeled with anti-NKp46 antibodies applied to tissues sections ex vivo (**Figure S2C**). Thus, both microscopy (**Figure 1A**) and intravascular staining (**Figure 1E, F**) revealed few WP-localized NK cells and a predominance of RP-localized NK cells in the absence of virus (**Figure 1F, Figure S1**). Following LCMV infection, the fraction (**Figure 1F**) and number (**Figure 1E**) of splenic iv-NKp46^{neg} NK cells increased over time, peaking at day 3 and returning to near baseline by day 6 of infection. Notably, differences in iv-NKp46 staining could not be explained by differences in ex vivo measurement of NKp46 expression levels (**Figure S2A**). In total, these results show that NK-

cells transiently re-locate to the WP during the first three days of LCMV infection, a window of time concomitant with perforin-dependent NK-cell killing of activated T cells (4).

The intravascular staining method permitted comparison of the phenotype of NK cells in the WP (iv-NKp46^{neg}) and RP (iv-NKp46^{+}) after infection. Each subset exhibited similar expression levels of activating (Ly49H, DNAM-1, NKG2D) and inhibitory (CD94, NKG2A, KLRG1) NK-cell receptors, although NKG2D expression was slightly reduced on WP NK cells (**Figure S3A-F**). Expression of CXCR3 (**Figure S3G**) as well as the distribution of immature (CD11b^{neg}CD27^{+}), transitional (CD11b^{+}CD27^{+}), and mature (CD11b^{+}CD27^{neg}) NK cell subsets (**Figure S3H**) were also similar between the RP and WP. However, WP NK cells expressed higher levels of granzyme B and the IL-2 receptor alpha (CD25) than RP NK cells (**Figure S3I-J**). Thus, WP NK cells may be more cytolytically active and IL-2 responsive than their counterparts in the RP.

Type 1 interferons are necessary and sufficient to drive NK cell localization in white pulp. In contrast to LCMV infection, vaccination with a replication incompetent adenovirus serotype 5 vector (Ad5) triggered robust T-cell responses without any evidence of T-cell suppression by NK cells (21). We hypothesized that the absence of NK-cell immunoregulatory functions after Ad5 vaccination may be associated with weak or absent induction of NK-cell localization within T-cell zones. As such, we immunized mice with replication incompetent Ad5 vectors harboring either the glycoprotein (GP) of LCMV (21) (**Figure 2A-B**) or β-galactosidase (Ad5-LacZ) (**Figure S4, Figure 2C-F**) and assessed NK-cell localization. There was no measurable localization of NK cells within the WP at any time point measured after Ad5 immunization (**Figure 2A-B, Figure S4A-B**), supporting an association between NK-cell migration and immune regulation.

One important distinction between LCMV infection and Ad5-vector immunization lies in the magnitude of type I interferon (IFN-I) responses, where LCMV is a potent inducer of IFN-I (22). Indeed, we measured a ~5-fold increase in *Ifna9* and *Infb1* expression during LCMV infection but not Ad5 immunization (**Figure 2C**). Thus, we hypothesized that robust IFN-I expression during LCMV infection but not after Ad5 immunization promotes NK-cell migration. Indeed, daily provision of recombinant IFN-alpha (rIFN-α) during Ad5 immunization resulted in increased NK-cell localization within the WP (**Figure 2D-E**) and T-cell zones (**Figure 2D-F**) of the spleen, as well as enhanced NK-cell accumulation in the draining lymph nodes (**Figure S5**). In agreement with the importance of IFN-I for NK-cell migration, addition of anti-IFN-αβ receptor (α-IFNAR-1) blocking antibodies during LCMV infection impeded lopalization of NK cells in the WP (**Figure 2G-H**) and T-cell zones (**Figure 2G-I**) of the spleen. IFN-I blockade also reduced NK-cell accumulation in draining lymph nodes after LCMV infection (**Figure S5**). Thus, IFN-Is are important triggers of NK cell migration to T-cell rich sites in lymphoid tissues.

NK cells require CXCR3 for splenic T-cell zone localization. Inflammatory recruitment of NK cells to lymph nodes during poxvirus infection or after dendritic cell immunization requires expression of the chemokine receptors CXCR3 on NK cells (17, 23, 24). Likewise, CXCR3 is implicated in NK-cell localization to the WP after polyinosinic:polycytidylic acid (poly I:C) injection or MCMV infection (14, 15). Therefore, we hypothesized that type I IFN induction of CXCR3 ligands and CXCR3 expression on NK cells is vital for migration to T-cell zones in the WP. The expression of the CXCR3 ligands *Cxcl9, Cxcl10* and *Cxcl11* were elevated following LCMV infection but not Ad5 immunization (**Figure 3A**). IFNAR-1 blockade dampened LCMV-mediated induction of *Cxcl10* and *Cxcl11* (**Figure S6**).

To address the role of CXCR3 in NK cell migration and suppressive function without undermining any role for CXCR3 in T-cell activation, we generated mixed bone marrow chimeric (BMC) mice harboring CXCR3-sufficient T and B cells in conjunction with an innate compartment that is either CXCR3-sufficient or –deficient. *Rag*^{KO}*Cxcr3*^{WT} or *Rag*^{KO}*Cxcr3*^{KO} bone marrow cells (Ly5.2) were mixed at a 9:1 ratio with wild-type bone marrow cells (Ly5.1) prior to reconstitution of lethally irradiated mice. This protocol (25) ensures RAG-dependent cells, including T and B cells, are ubiquitously CXCR3-sufficient while innate cells like NK cells are predominately derived from the *Rag*^{KO} precursors that are either *Cxcr3*-sufficient or -deficient. We confirmed that CXCR3 was absent from NK cells in the CXCR3^{KO} chimeras (**Figure S7A**). The resulting CXCR3^{KO}- and CXCR3^{WT}-chimeric mice were infected with LCMV prior to assessment of NK-cell localization on day 3 of infection (**Figure 3B**). Similar to infected non-chimeric C57BL/6 mice (WT), a substantial fraction of NK cells co-localized within the WP (**Figure 3C**) and T-cell zones (**Figure 3D**) of infected CXCR3^{WT} BMC mice. In contrast, NK-cell frequencies within the WP (**Figure 3C**) and T-cell zones (**Figure 3D**) were significantly reduced in CXCR3^{KO} BMCs. Thus, localization of NK cells within T-cell rich regions of the WP during LCMV infection depends on NK-cell expression of CXCR3.

CXCR3 is required for NK-cell suppression of anti-viral T cells. Transient localization of NK cells in the WP during LCMV infection (**Figure 1**) coincides with the window of time during which NK cells kill activated T cells (4). Since CXCR3 is necessary for NK-cell localization within T-cell rich WP during LCMV infection (**Figure 3**), we hypothesized that mice lacking CXCR3 on NK cells would display an enhanced virus-specific T-cell responses similar to mice depleted of NK cells. Anti-NK1.1 antibody treatment one day before infection effectively depleted NK cells from both sets of mixed BMC mice (**Figure S7B**). At day 7 of LCMV infection, antiviral T-cell responses

were assessed by intracellular cytokine staining after in vitro re-stimulation with viral peptide. The proportion (**Figure 4A**) and number (**Figure 4B**) of IFN-γ^+TNF$^+$ LCMV GP_{64-80}-specific CD4 T cells was elevated to a similar extent in CXCR3^{KO} chimeras and in NK-cell depleted CXCR3^{WT} chimeras, with no apparent additive effect of NK cell-depletion in CXCR3^{KO} chimeras. The numbers of splenic Fas$^+$GL7$^+$ germinal center B cells were similarly increased by CXCR3-deficiency or NK-cell depletion (**Figure 4C**).

As an alternative test of our hypothesis, CXCR3-sufficient LCMV-specific TCR transgenic SMARTA CD4 T cells (Ly5.1) were seeded in either CXCR3-sufficient C57BL/6 mice or germline *Cxcr3^{KO}* mice (Ly5.2) prior to NK-cell depletion and LCMV inoculation. On day 6 of infection, the frequencies of donor SMARTA cells expressing IFN-γ and TNF were increased in *Cxcr3^{KO}* and NK-cell-depleted recipient mice relative to those in control animals (**Figure 4D**). There was no additional effect of NK-cell depletion in *Cxcr3^{KO}* hosts. Thus, CXCR3 expression is important for NK-cell suppression of antiviral T cells.

These data reveal a crucial spatiotemporal mechanism of NK-cell regulation of antiviral T-cells. We identify CXCR3 as essential for both transient localization of NK cells within T-cell zones after infection and in the resulting suppression of antiviral T-cells by NK cells. We demonstrate that IFN-I-dependent induction of CXCR3 ligand expression is vital mechanism determining NK-cell localization in T-cell zones. Adenoviral vectors that do not robustly induce IFN-I also fail to elicit NK-cell follicular migration and associated suppression of T cells. Therefore, CXCR3 directs crucial redistribution of NK cells during inflammation that brings these cells into close proximity of activated T cells to permit perforin-dependent regulation (**Figure S8**). Translational targeting of this mechanism represents an innovative means of potentially enhancing vaccine efficacy and boosting antibody generation.

The transient nature of WP localization of NK cells represents a mechanistic explanation for the narrow temporal window of immunoregulatory function of NK cells. Measurable killing of activated T cells by NK cells is limited to the first three days of acute LCMV infection (3, 4), overlapping with the peak localization of NK cells in the WP reported here. Rapid egress or loss of NK cells from T-cell zones after day 3 likely limits further immunoregulatory killing. The degree to which different pathogens or vaccines trigger CXCR3-dependent migration of NK cells is linked to the ability to induce IFN-I responses. Some adenoviral vectors (e.g. Ad28 and Ad35) do trigger IFN-I release (26), but these responses are several orders of magnitude lower than those seen after LCMV infection (22) and rapidly wane within hours of inoculation.

In addition to LCMV, re-localization of NK cells to T-cell zones (14-16) and NK-cell suppression of T cells are also triggered during infections with MCMV or after injection of TLR ligands (4). Induction of IFN and ligands for CXCR3 (27) are shared features of these contexts. This explains why viruses like vaccinia virus and Ad5 vectors that weakly induce IFN responses (26, 28) also poorly stimulate NK-cell inhibition of T cells (21, 29). Of note, transient blockade of IFN-I early during LCMV infection can enhance antiviral T- and B-cell responses (30). Ablation of the IFNAR on NK cells exerts a similar effect (31). In MCMV and SIV/HIV infections, where NK cells exhibit potent antiviral function, this migration of NK cells to T-cell zones serves to protect these tissues from virus (15, 19). However, conservation of this migration during immunization or during infection with viruses that are refractory to NK-cell antiviral functions (e.g. LCMV) is more consequential for immune regulation than for immune defense.

Our discovery of CXCR3-mediated, WP localizing NK cells in suppression of T cells opens new avenues of research that will enhance our understanding of the biology of this process as well as provide translational targets to circumvent this activity of NK cells.

Immunosuppressive function of NK cells is linked to reduced T-cell memory (3), neutralizing antibody titers (3, 8), antibody affinity maturation (6), and vaccine efficacy (10). Our discovery highlights the potential for interventions blocking CXCR3 or its ligands in NK-cell immunosuppression to enhance vaccine efficacy. Further characterization of WP localized NK cells is likely to reveal key mediators involved in T-cell suppression. Indeed, WP and RP localized NK cells exhibit markedly different transcriptomes (unpublished observations). This represents a promising pipeline for identification of translational targets to enhance vaccine-elicited immune responses by circumventing NK-cell activity.

METHODS

Study approval. Experiments were performed according to ethical guidelines approved by the Institutional Animal Care and Use Committee and the Institutional Biosafety Committee of Cincinnati Children's Hospital Medical Center. Additional methods details are presented in the Supplemental Material.

AUTHOR CONTRIBUTIONS

Conceptualization and design of study (AA, MTM, SNW), performance of experiments and data acquisition (AA, LMC, HAF, HC, MTM, JT, NL, SR, HS, DK), data analysis (AA, LMC, HAF, HC, MTM, JT, NL, SR, SNW), provision of reagents (EB, DB), drafting of the manuscript (AA, SNW), and critical editing of the manuscript (AA, LMC, HAF, HC, MTM, JT, NL, SR, HS, DK, EB, DB, SNW).

ACKNOWLEDGEMENTS

We thank Dr. Kofron and Comprehensive Mouse and Cancer Core for support, Dr. Molkentin for Ad-LacZ, Dr. Crotty for SMARTA mice, Shane D'Souza for crafting the summary model, and Drs. Kottyan, Lewkowich, Hildeman, Herro, and Borchers for critical reading of the manuscript.

Funding for this work was provided by NIH grants DA038017, AI148080, AR073228 (SNW), T32GM063483 (AA, NL, HAF), and T32AI118697 (AA). Support was also provided by the Cincinnati Children's Research Foundation (SNW), L.B. Research and Education Foundation (N.L.), and Albert J. Ryan Foundation (AA). The Flow Cytometry Core was supported by NIH grants AR070549 and DK078392.

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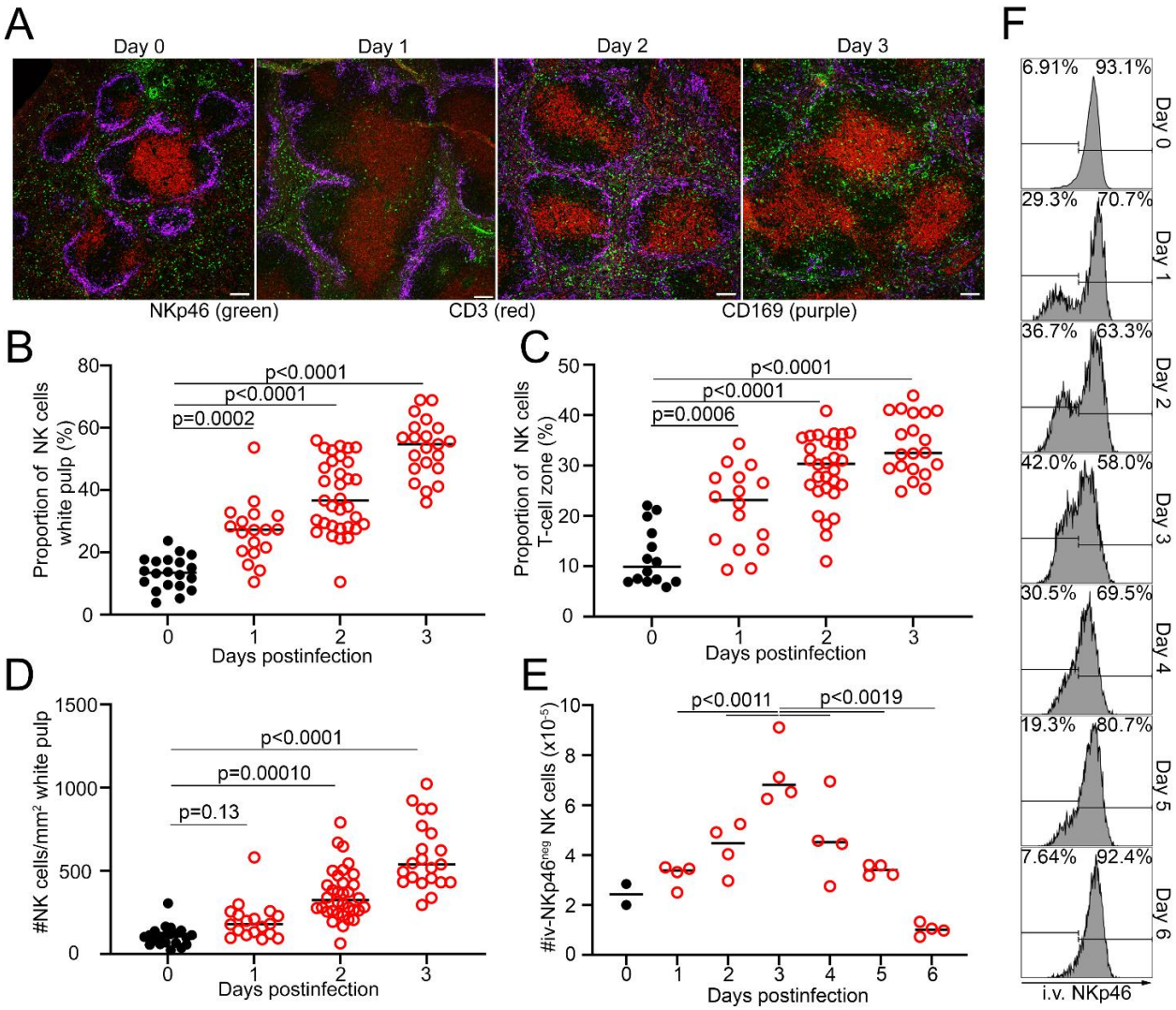

Figure 1. Transient positioning of NK cells in T-cell zones during infection. (A-F) C57BL/6 mice (n=3-4/group) were infected with the Armstrong strain of LCMV. Proportion (B, C) and number (D) of NKp46^{+} NK cells (green) enumerated within (B, D) CD169 macrophage (purple) bound WP or in (C) CD3^{+} T-cell zones (red) is plotted (5-12 follicles/mouse). (E, F) Mice were intravenously injected at indicated times points with anti-NKp46 antibody 3 minutes prior to euthanasia to label splenic NK cells (CD3^{neg}TCRβ^{neg}CD8α^{neg}CD49b^{+}NK1.1^{+}ex-vivoNKp46^{+}) in RP (iv-NKp46^{+}) or WP (iv-NKp46^{neg}) regions. (E) Mean number of iv-NKp46^{neg} NK cells is graphed (4 mice/time point). (F) Representative histograms of iv-NKp46 staining of gated NK cells. Data representative of two independent experiments with statistical differences determined by one-way ANOVA. Scale bars measure 100 μm.

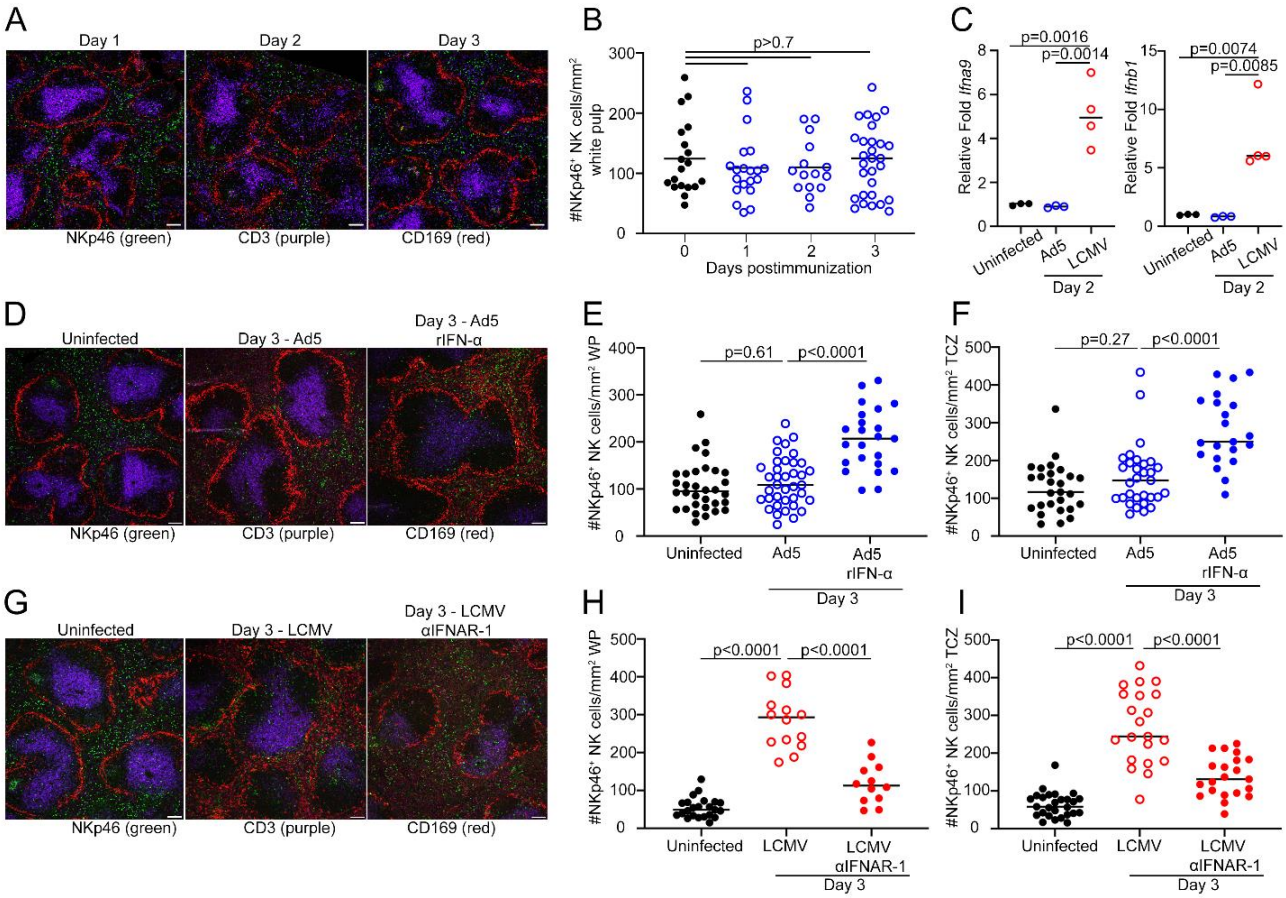

Figure 2. Type 1 interferons are necessary and sufficient to drive NK cell white pulp localization. C57BL/6 mice were inoculated with (A-B) Ad5-GP, (C-F) Ad5-LacZ, or (C, G-I) LCMV. One group (D-F) of Ad5-LacZ inoculated C57BL/6 mice were treated with 8 μg/day recombinant IFN-α while another group (G-I) of LCMV-infected C57BL/6 mice were treated with 400 μg/day anti-IFNAR-1 antibody. At indicated times points, spleens were imaged (A, D, G) to determine localization of NKp46$^+$ NK cells (green) relative to CD3$^+$ T cells (purple) and CD169$^+$ macrophages (red), with enumeration of NK cells in (B, E, H) CD169-delineated WP or (F, I) CD3$^+$ T-cell zones (n=3-4 mice, 5-12 follicles/mouse). (C) Relative expression of *Ifna9* and *Ifnb1* two days following LCMV infection or Ad5-LacZ inoculation compared to uninfected mice. Data representative of two independent experiments with statistically significant differences determined by one-way ANOVA. Scale bars measure 100 μm.

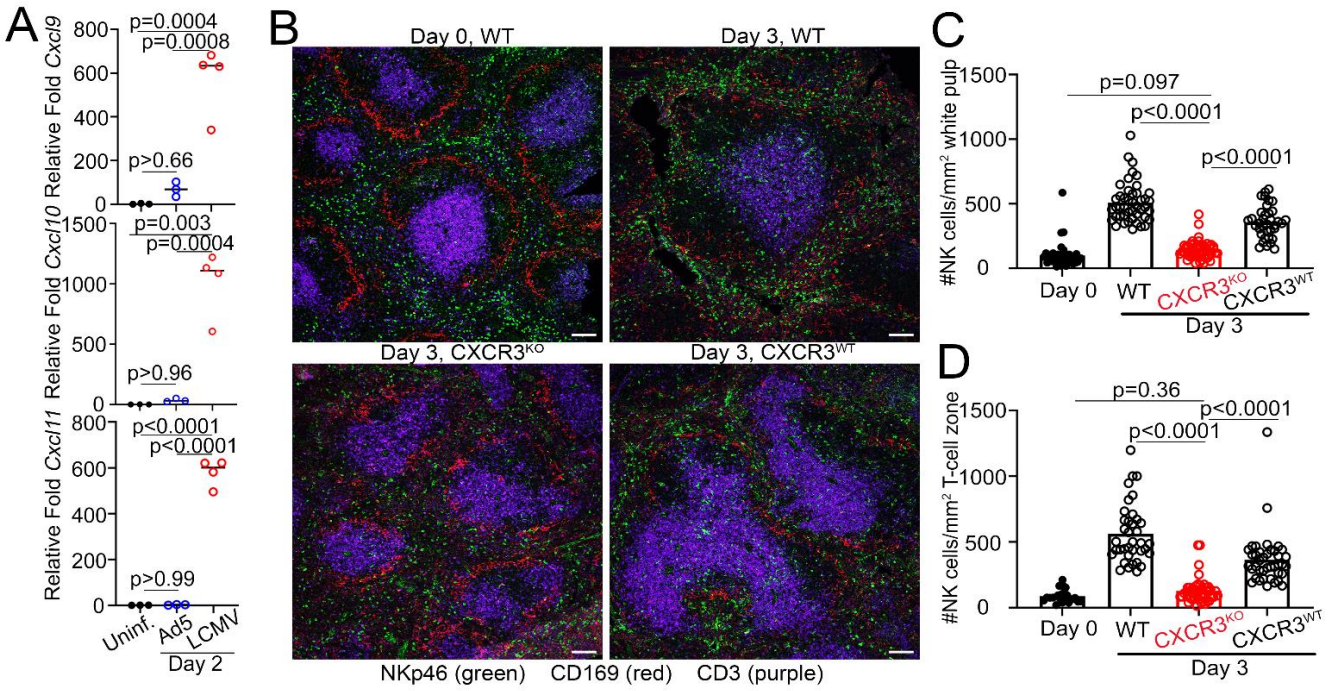

Figure 3. NK cells require CXCR3 for splenic T-cell zones localization. (A) Relative expression of *Cxcl9, Cxcl10 and Cxcl11* in the spleens of uninfected, Ad5-LacZ inoculated or LCMV-infected C57BL/6 mice (n=3-4/group). (B-D) C57BL/6 mice (WT) and mixed CXCR3^{WT} or CXCR3^{KO} bone-marrow chimeras (n=3) were infected with LCMV. Prior to (Day 0) or after (Day 3) infection, (B) confocal microscopy was used to determine median number (5-12 follicles/mouse) of NKp46^{+} NK cells (green) in (C) CD169^{+} macrophage (red) bordered WP or in (D) CD3^{+} T-cell zones (purple). Data representative of two independent experiments with statistically significant differences determined by one-way ANOVA. Scale bars measure 100 µm.

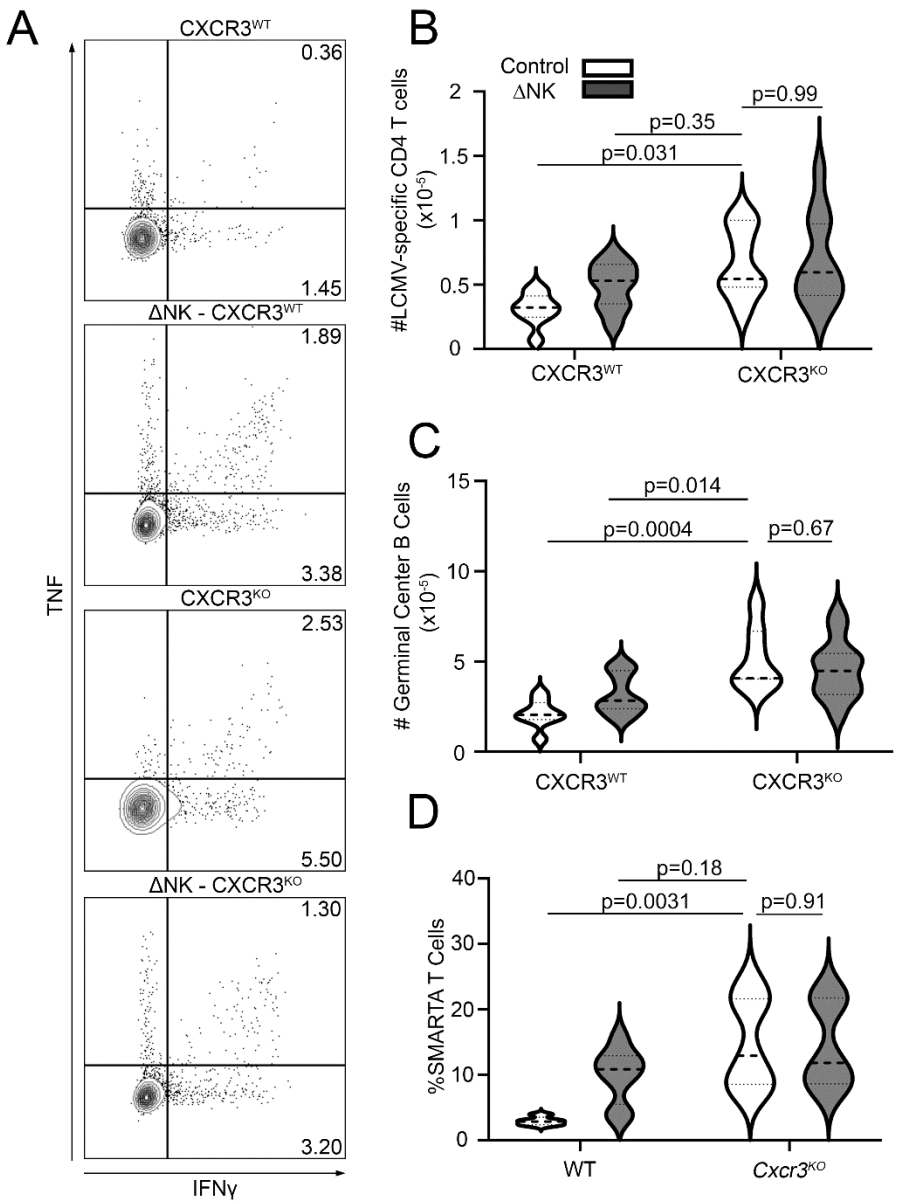

Figure 4. CXCR3 is required for NK-cell suppression of antiviral T cells. (A-C) Mixed CXCR3^{WT} and CXCR3^{KO} bone-marrow chimeric mice (n=7-12 mice/group) were depleted of NK cells via anti-NK1.1 (ΔNK) or control antibody one day prior to LCMV infection. (B) Numbers of IFN-γ^+ TNF$^+$ expressing LCMV GP_{64-80}-specific CD4 T cells were measured on day 7 by intracellular cytokine staining and flow cytometry. (C) Numbers of Fas$^+$ GL7$^+$ germinal center B cells were measured. (D) C57BL/6 and *Cxcr3^{KO}* (n=3-4 mice/group) mice were depleted or not of NK cells (ΔNK) prior to intravenous infusion of $5x10^5$ LCMV-specific Ly5.1$^+$ transgenic SMARTA CD4 T cells and infected one day later with LCMV. At day 6, the proportions of donor TCR-Vα2$^+$Ly5.1$^+$CD4$^+$ SMARTA T cells were quantified in spleens of recipient mice. Statistical analyses were performed using one-way ANOVA. Data pooled from two independent experiments.