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Immunity to infection

Basic

Research Article

Non-neutralizing antibodies protect against chronic LCMV infection by promoting infection of inflammatory monocytes in mice

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Antibodies play an important role in host defense against microorganisms. Besides direct microbicidal activities, antibodies can also provide indirect protection via crosstalk to constituents of the adaptive immune system. Similar to many human chronic viral infections, persistence of Lymphocytic choriomeningitis virus (LCMV) is associated with compromised T- and B-cell responses. The administration of virus-specific non-neutralizing antibodies (nnAbs) prior to LCMV infection protects against the establishment of chronic infection. Here, we show that LCMV-specific nnAbs bind preferentially Ly6C^{hi} inflammatory monocytes (IMs), promote their infection in an Fc-receptor independent way, and support acquisition of APC properties. By constituting additional T-cell priming opportunities, IMs promote early activation of virus-specific CD8 T cells, eventually tipping the balance between T-cell exhaustion and effector cell differentiation, preventing establishment of viral persistence without causing lethal immunopathology. These results document a beneficial role of IMs in avoiding T-cell exhaustion and an Fc-receptor independent protective mechanism provided by LCMV-specific nnAbs against the establishment of chronic infection.

Keywords: LCMV · Chronic viral infection · Inflammatory monocytes · Non-neutralizing antibodies · T-cell activation

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Chronic infections with noncytopathic viruses, such as HIV, hepatitis B or C viruses affect several hundred million people worldwide. The high incidence and mortality of these infections highlights the importance of studies devoted to better understand chronic diseases and of development of potent antiviral treatments. Viruses have evolved different strategies to evade the immune system and persist in the host. Together, the location, timing, and magnitude of the immune response combined with the speed of virus replication, its cytopathogenicity, and spread determine the fate of the viral infection.

Despite the fact that antiviral vaccines aim at producing primarily neutralizing antibodies (nAb), there is a growing body of evidence emphasizing the protective potential of non-neutralizing antibodies (nnAbs) against infection with different viruses [1] including HIV [2], influenza virus [3, 4], Ebola virus [5], CMV [6],

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vaccinia virus [7], Lymphocytic choriomeningitis virus (LCMV) [8, 9], and others.

Usually, nnAbs mediate protection through the interaction of the Fc-domain with different Fc-receptors [10], triggering antibody-dependent cellular cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP), complement-dependent cytolysis (CDC), or (steric) inhibition of viral proteins important for replication, assembly, and release [1, 2, 11]. In case of LCMV, earlier work by Hangartner and Bergthaler et al. demonstrated that the presence of nnAbs at the time of infection limited early viral spread and prevented the establishment of chronic infection [12, 13].

A few recent studies propose alternative protective mechanisms mediated by nnAb independent of the commonly described Fc gamma receptors (FcRγ). For example, FcRn is part of the endolysosomal system of most epithelial and hematopoietic cells where it regulates the intracellular fate of both IgG and IgG-containing immune complexes [14]. Additionally, the tripartite motif-containing receptor 21 (Trim21) acts as a very high-affinity intracellular antibody receptor that recognizes nonenveloped antibody-coated viruses, such as Adenovirus, and inhibits viral replication [15–17]. In the case of LCMV, two more recent studies confirmed nnAb-mediated protection from establishment of chronic infection and both excluded an involvement of either complement or binding to FcRγ for the protective mechanism, instead emphasizing the requirement of $CD8^+$ T cells for viral clearance [8, 9].

Here, we show that LCMV-specific nnAbs protect against establishment of chronic viral infection by binding predominantly $Ly6C^{hi}$ inflammatory monocytes (IMs). In this way, these nnAbs strongly promoted infection of IMs in an FcRγ-independent manner and lead to acquisition of an activated APC phenotype by upregulating CD11c, MHC, and costimulatory molecules. IMs from infected mice treated with LCMV-specific nnAbs served as additional APCs and boosted early activation of virus-specific T cells, leading to improved effector phenotype differentiation and enhanced cytotoxicity, thereby preventing the establishment of chronic infection.

Considering the growing interest and importance of nnAbs as an alternative protection against persistent viral infections, defining their protective mechanisms contributes to the advance in the search of preventive and therapeutic treatments against chronic viral infections.

Results

LCMV-specific nnAbs protect against chronic infection

We used LCMV infection as a well-established murine model for a chronic virus infection to study the mechanism by which nnAbs prevent viral persistence [8, 9]. Mice infected with either low- (inducing acute infection) or high-dose (inducing chronic infection) LCMV clone 13 for 12 days develop LCMV-specific binding antibodies that lack neutralizing activity [8, 9]. We have used either serum from such LCMV-infected mice or the monoclonal LCMV-specific nnAb KL53 to investigate the protective mechanism of nnAbs in chronic LCMV infection.

First, we confirmed that day 12 immune serum (IS) and the monoclonal Ab KL53 exhibited binding activity toward LCMV-derived proteins, demonstrated by specific staining of LCMV infected cells (Supporting information Fig. S1A). We also confirmed lack of neutralizing activity against LCMV in day 12 IS (Supporting information Fig. S1B). Next, we assessed the effects of transfused nnAbs on the course of a chronic LCMV infection. For this, we opsonized 2×10^6 viral particles of LCMV with 250 μl IS and infected WT mice. Within the first week of infection, both mice infected with IS-opsonized virus as well as control mice harbored comparable viral loads (Supporting information Fig. S1C). After 8 days, however, mice that had received IS-opsonized virus started to clear the infection and 12 days postinfection (dpi) all IS-treated mice had controlled the infection (Supporting information Fig. S1C). These data are in line with previous reports [8, 9]. Importantly, IS was protected against the establishment of chronic LCMV infection in the absence of FcRγ or $CD4^+$ T cells (Supporting information Fig. S1D).

We hypothesized that opsonization of LCMV might influence its in vivo tropism. To get a broad overview on LCMV distribution in secondary lymphoid organs, we analyzed spleens from various time points after infection in presence or absence of IS by confocal microscopy. As previously described [18,19], LCMV was contained in the marginal zone of the spleens of both groups within the first day of infection. At 3dpi, LCMV had spread throughout the white pulp in both groups. One week postinfection, the virus was still detectable in all spleens of infected mice. However, by 8 and 12dpi, mice infected with IS-opsonized LCMV had completely eliminated LCMV, whereas the virus was still strongly detectable in control mice (Supporting information Fig. S1E), confirming the quantification of viral particles by focus forming assays (Supporting information Fig. S1C). Taken together, the gross anatomical distribution of LCMV in the spleen early during infection seemed to be unaltered in presence or absence of nnAbs.

LCMV infects nonhematopoietic cells including fibroblastic reticular cells. Cytotoxic T-cell-mediated killing of infected cells results in destruction of the structure of secondary lymphoid organs, defined by distinct B- and T-cell zones [20]. In this regard, we evaluated the effects of enhanced viral clearance on the splenic architecture as a measure of immunopathology. We stained spleen sections from mice infected with high-dose LCMV with or without IS at the peak of the response 8dpi with antibodies specific for CD169 for the marginal zone, antibodies against CD3 and B220 for T- and B-cell zone, respectively (Supporting information Fig. S1F). We evaluated the disruption of the splenic architecture by the infiltration of T cells ($CD3^+$) in the B-cell zones ($B220^+$) of spleens of infected mice. The splenic structure was similarly compromised in mice infected with IS-opsonized LCMV compared to control mice as measured by the presence of $CD3^+$ cells in $B220^+$ regions.

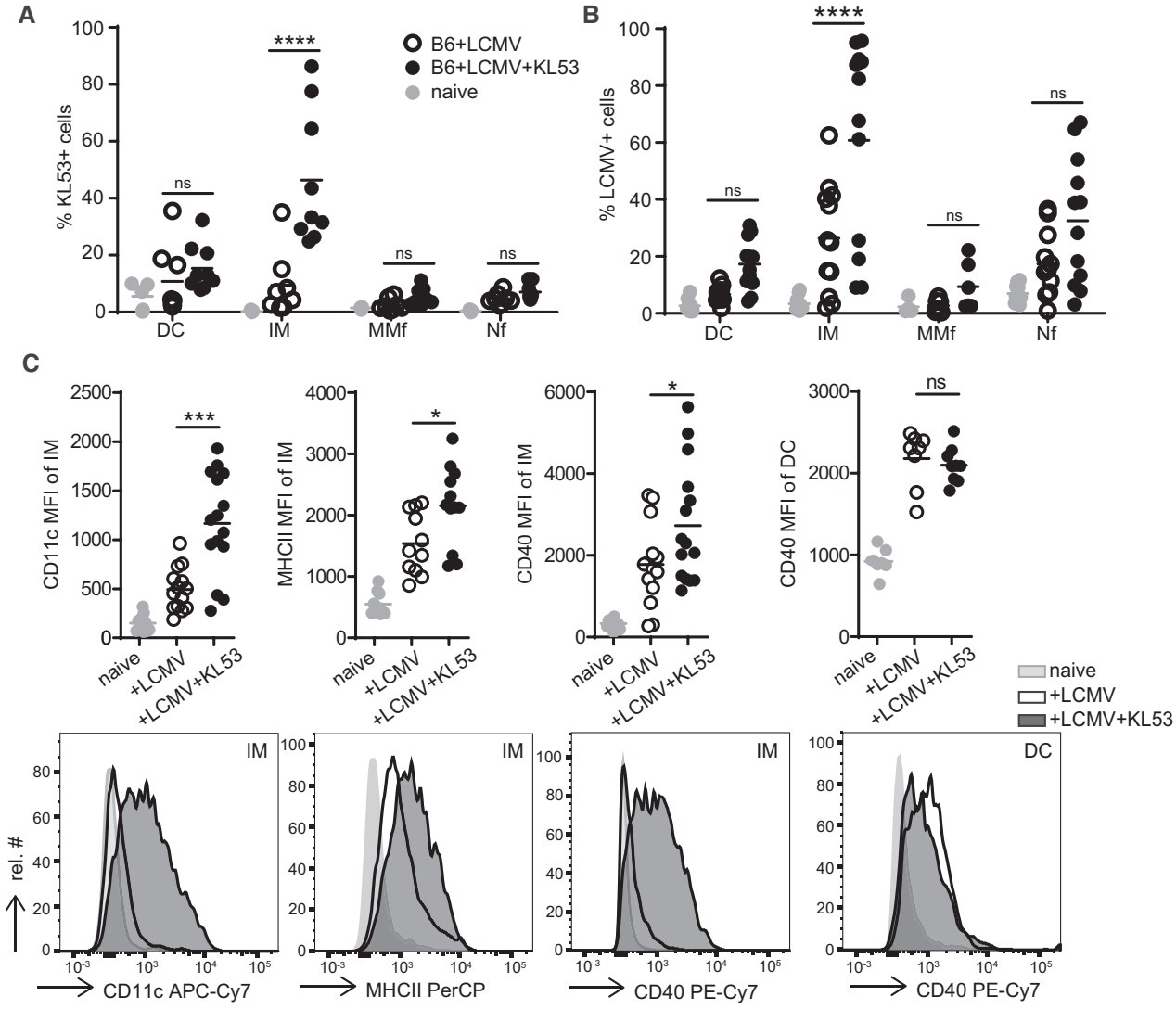

Figure 1. nnAbs bind predominantly inflammatory monocytes. WT mice were infected with LCMV with or without 0.5 mg KL53. (A) KL53$^+$ cells detected by surface staining of KL53 with anti-IgG2a, (B) LCMV$^+$ (VL4$^+$) cells detected by intracellular staining of LCMV NP by VL4 antibody, or (C) MFI of CD11c, MHCII, or CD40 in IMs or DCs in the spleens of naïve (grey), LCMV-infected (open circles), or LCMV-infected mice with KL53 (black circles) were determined 1dpi by flow cytometry, gated on live single cells, DCs (CD11c$^+$MHCII^{hi}), IM (CD11b$^+$Ly6C^{hi}), MMf (CD169$^+$), and Nf (CD11b$^+$Ly6C^{int}). Data of three independent experiments were pooled with n = 3 mice per group. Horizontal line represents the mean. Statistical analysis was performed using two-tailed unpaired Student's *t*-test, $^{****}p < 0.0001$.

LCMV-specific nnAbs promote APC maturation of Ly6C^{hi} inflammatory monocytes

Next, we analyzed the splenic hematopoietic cell types that had bound the nnAb KL53 in vivo 1 day after infection. Mice were infected with LCMV in presence or absence of KL53 and 1 day later splenocytes were analyzed for cell-surface bound KL53 using flow cytometry. Specifically, we evaluated the percentages of KL53$^+$ cells among CD11c^{hi}MHCII^{hi} DC, Ly6C^{hi}CD11b$^+$ IMs, CD169$^+$ metallophilic macrophages (MMf), and CD11b$^+$Ly6C^{int} neutrophils (Nf). Interestingly, KL53 preferentially bound to Ly6C^{hi} IMs and not to any other analyzed cell type (Fig. 1A). We confirmed the preferential binding of LCMV-specific nnAbs to Ly6C^{hi} IMs using polyclonal IS from LCMV-infected congenic IgH^a mice (Supporting information Fig. S2A). Additionally, we stained the same splenic populations for active infection with LCMV which revealed IMs as the cells with the highest viral tropism in the spleen (Fig. 1B). Thus, increased KL53 binding was found on highly infected cells. This correlation might be due either to KL53 recognition of already infected cells, shifted viral tropism of opsonized viral particles, or both. In order to exclude the role of FcRγ in the antibody-binding specifically on IMs, we incubated splenocytes from infected WT or FcRγ$^{-/-}$ with KL53 and measured its ability to bind IMs in FcRγ-independent way. We found similar amounts of IgG$^+$ IMs from both WT and FcRγ$^{-/-}$ mice (Supporting information Fig. S2B).

Next, we asked whether and how the binding of nnAbs on Ly6C^{hi} IMs affected their phenotype. We examined splenic

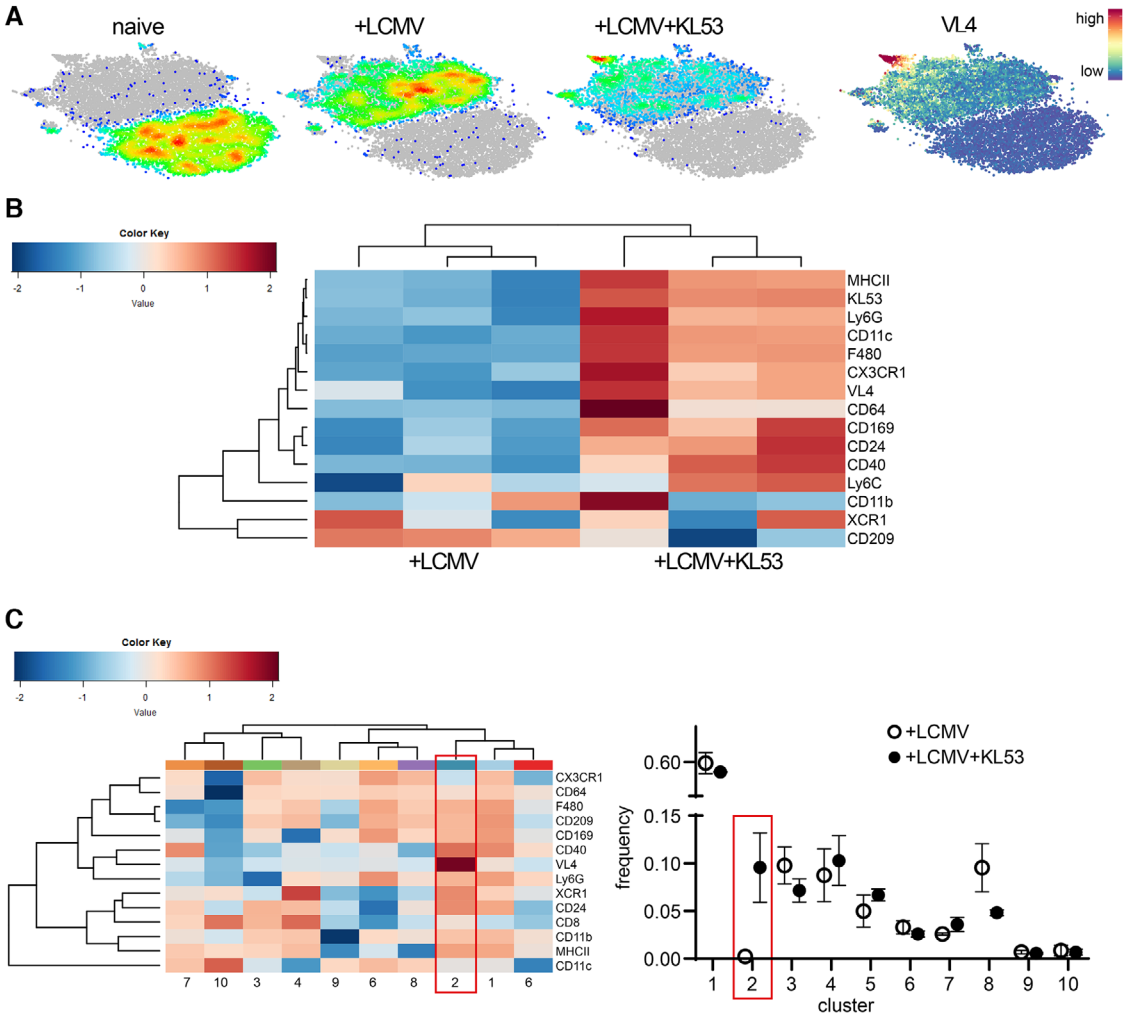

Figure 2. LCMV-specific nnAbs alter the IM phenotype. WT mice were infected with LCMV or opsonized LCMV with 0.5 mg KL53. Splenocytes were analyzed 1dpi by flow cytometry. (A) tSNE plots of pooled cells, gated on single, live, $CD45^+$, Lin^- (CD3, NK1.1, CD19, Ly6G) $CD11b^+Ly6C^{hi}$ inflammatory monocytes (gray). Color gradient represents distribution of cells isolated from infected mice (+LCMV), mice infected with KL53-opsonized virus (+LCMV+KL53), naïve (naïve) or VL4+ cells. (B) Heatmap showing medians of measured markers or percentages of $KL53^+$ inflammatory monocytes in mice infected with LCMV or KL53-opsonized LCMV 1dpi. Dendograms show hierarchical clustering of mice (columns) or markers (rows). (C) Clustering of inflammatory monocytes isolated from mice infected with LCMV or KL53-opsonized LCMV 1dpi. Heatmap of marker medians of the clusters generated by FlowSOM. Dendograms show hierarchical clustering of clusters (columns) or markers (rows). Cluster frequency ±SEM in mice infected with LCMV or KL53-opsonized LCMV. Data are representative of two independent experiments with n = 3 per group.

$CD11b^+Ly6C^{hi}$ monocytes 1dpi in the presence or absence of LCMV-specific nnAb KL53. LCMV infection in absence of KL53 already increased CD11c, MHCII, and CD40 expression within $CD11b^+Ly6C^{hi}$ monocytes to some extent, but treatment with KL53 boosted the expression of these markers significantly in $Ly6C^{hi}$ monocytes compared to infected mice in the absence of KL53 (Fig. 1C). Interestingly, the KL53-induced APC maturation was selective for $Ly6C^{hi}$ monocytes, as CD40 expression on DCs was comparable in presence or absence of KL53 (Fig. 1C).

As we identified IMs to be particularly affected by treatment with LCMV-specific antibodies early during infection, we further examined their phenotype in response to LCMV opsonized with virus-specific KL53 mAb. We performed a cluster analysis based on an extended panel of myeloid markers on IM populations 1dpi from naïve, LCMV-infected, and mice-infected KL53-opsonized LCMV. tSNE plots depict IMs from the three groups as distinct populations (Fig. 2A). While IMs from naïve mice show hardly any overlap with IMs from infected mice, IMs from LCMV-infected mice share common characteristics. Yet, IMs from mice treated with KL53-opsonized LCMV cluster as a distinct population that colocalizes with the highest abundance of $VL4^+$ LCMV-infected cells (VL4 being an LCMV NP-specific antibody used for intracellular staining of LCMV infected cells). IMs from KL53-treated mice showed higher levels of a series of APC-related markers (such as MHCII, CD11c, CX3CR1, CD40, etc.) as compared to IMs from infected mice in the absence of antibodies, as depicted by a heatmap in Fig. 2B. We have performed a further clustering analysis of IMs from infected mice using FlowSOM. We have chosen

10 clusters with distinct expression levels of myeloid- and APC-related markers and we observed various frequencies of IM populations from infected mice treated or not with KL53 within each cluster (Fig. 2C). Notably, cluster 2 is characterized by high levels of VL4 binding and is exclusively present in IMs of mice treated with KL53. It also shows high expression of the other APC-related markers (such as XCR1, MHCII, CD40) and suggests that infection itself might affect the phenotype of monocytes and that nnAbs enhance this effect by redirecting infection towards IMs.

LCMV-specific nnAb enhance infection of IMs in an FcRγ-independent manner

NnAbs can shift the viral tropism towards hematopoietic cells and specifically professional APCs through the recognition by Fc-receptors [21–23]. However, the protective mechanism mediated by LCMV-specific nnAbs was shown to be FcRγ-independent [8, 9]. Therefore, we tested whether LCMV-specific nnAbs would enhance viral tropism toward hematopoietic cells in an FcRγ-independent manner. WT or FcRγ$^{-/-}$ mice were infected with LCMV with or without IS. Three dpi, LCMV-infected cells were quantified in splenocytes by intracellular staining for LCMV NP. Both WT and FcRγ$^{-/-}$ mice showed a significant increase in infection of splenocytes and most prominently of CD11b^{+} splenocytes in presence of IS (Fig. 3A). Importantly, presence of IS did not show any effect on the infection of nonhematopoietic cells, such as lymphoid stroma or lung epithelial cells, as early as 3dpi. However, at later time points (6dpi), a reduced frequency of infected nonhematopoietic cells was observed in mice infected with IS-opsonized LCMV (Supporting information Fig. S3).

Additionally, we analyzed the effects of IS on the infection of DCs. While IS did not affect the numbers of DCs in infected mice, it significantly increased the percentages of infected DCs in WT but not in FcRγ$^{-/-}$ mice (Fig. 3B). Therefore, LCMV-specific nnAbs specifically promoted infection of DCs in an FcR-dependent manner, while they enhanced infection of CD11b^{+} cells in an FcR-independent manner.

Next, we evaluated the infection of Ly6C^{hi} IMs in presence or absence of IS 3dpi (Fig. 3C), of KL53 and control IgG2a antibody (Supporting information Fig. S2C and S2D). Similar to DCs, IS did not affect the percentages and numbers of IMs, but dramatically increased the infection of these cells from 50 to more than 90%, and this effect was largely FcRγ-independent (Fig. 3C). Importantly, this effect was dependent on LCMV-specific antibodies, since LCMV opsonized with control IgG2a antibody did not enhance IM infection (Supporting information Fig. S2D). In addition, preferential infection of IMs was observed not only with nnAb-opsonized LCMV, but also when mice were pretreated with IS 1 day prior LCMV infection (Supporting information Fig. S2E). Together, these results indicate that Ly6C^{hi} IMs bound LCMV-specific nnAbs, acquired an activated APC phenotype and were preferentially infected by LCMV in presence of nnAbs, suggesting that they might act as additional APCs for the activation of LCMV-specific T cells.

IMs from IS-treated mice infected with LCMV enhance virus-specific T-cell proliferation

Based on the observation that IMs acquired an activated APC phenotype after LCMV infection in presence of IS, we hypothesized that they might promote T-cell priming and effector responses. To test this, we performed an in vitro experiment in which we supplemented DC-CD8^{+} T-cell cocultures with sorted Ly6C^{hi} IMs from mice that had been infected with LCMV in presence or absence of IS. DCs were sorted from day 3 LCMV-infected mice and cocultured with naïve CTV-labeled LCMV gp$_{33-41}$-specific TCR transgenic CD8^{+} T cells (P14). Of note, DCs were used directly ex vivo without additional antigen pulsing. We analyzed the extent of activation and proliferation of CTV^{+} P14 cells 3 days later by flow cytometry. While very few P14 cells diluted CTV and upregulated CD44 when primed with DCs from LCMV- infected mice in the presence of Ly6C^{hi} IMs from naïve mice, a significant percentage of P14 cells proliferated and upregulated CD44 when cultures were supplemented by Ly6C^{hi} IMs from LCMV-infected mice (Fig. 4A). Remarkably, when DC-P14 cocultures were supplemented with IMs from IS-treated LCMV-infected mice, P14 cells exhibited significantly stronger proliferation compared to control cultures (Fig. 4A). Importantly, Ly6C^{hi} IMs from LCMV-infected mice did not affect the activation of OVA-specific OT-I cells (Supporting information Fig. S4A), demonstrating that Ly6C^{hi} IMs boosted T-cell activation and proliferation in an antigen-dependent manner.

Next, we asked whether Ly6C^{hi} IMs from IS-treated LCMV-infected mice would also promote CD8^{+} T-cell priming in vivo. To address this question, we generated IMs in LCMV-infected mice in the presence or absence of IS for 3 days. We then sorted and transferred these IMs into LCMV-infected CCR2$^{-/-}$ mice that lack circulating IMs which had received P14 T cells. Seven days post-transfer, we quantified virus-specific P14 T cells in the blood of infected mice (Fig. 4B). We found significantly more P14 cells in mice that received IMs from IS-treated LCMV-infected mice. This data indicate that IMs that originate from LCMV-infected mice in the presence of nnAbs were able to enhance virus-specific T-cell accumulation in vivo.

LCMV-specific nnAbs increase virus-specific T-cell responses

It was previously shown that nnAb-supported control of chronic LCMV infection involves CD8^{+} T cells [9]. We corroborate this finding by showing that CD4^{+} T cells are dispensable for IS/nnAb-promoted control of chronic LCMV infection (Supporting information Fig. S1D). We therefore analyzed the LCMV-specific CD8^{+} T-cell response in LCMV-infected mice in the presence or absence of IS (Fig. 5) or monoclonal nnAb KL53 (Supporting information Fig. S4C) at 7dpi, when the viral loads were still comparable between IS-treated and untreated mice (Supporting information Fig. S1C [8, 9]). The percentages of endogenous total CD44^{hi} T cells and LCMV gp$_{33-41}$-specific CD8^{+} T cells within the CD8^{+} T-cell pool

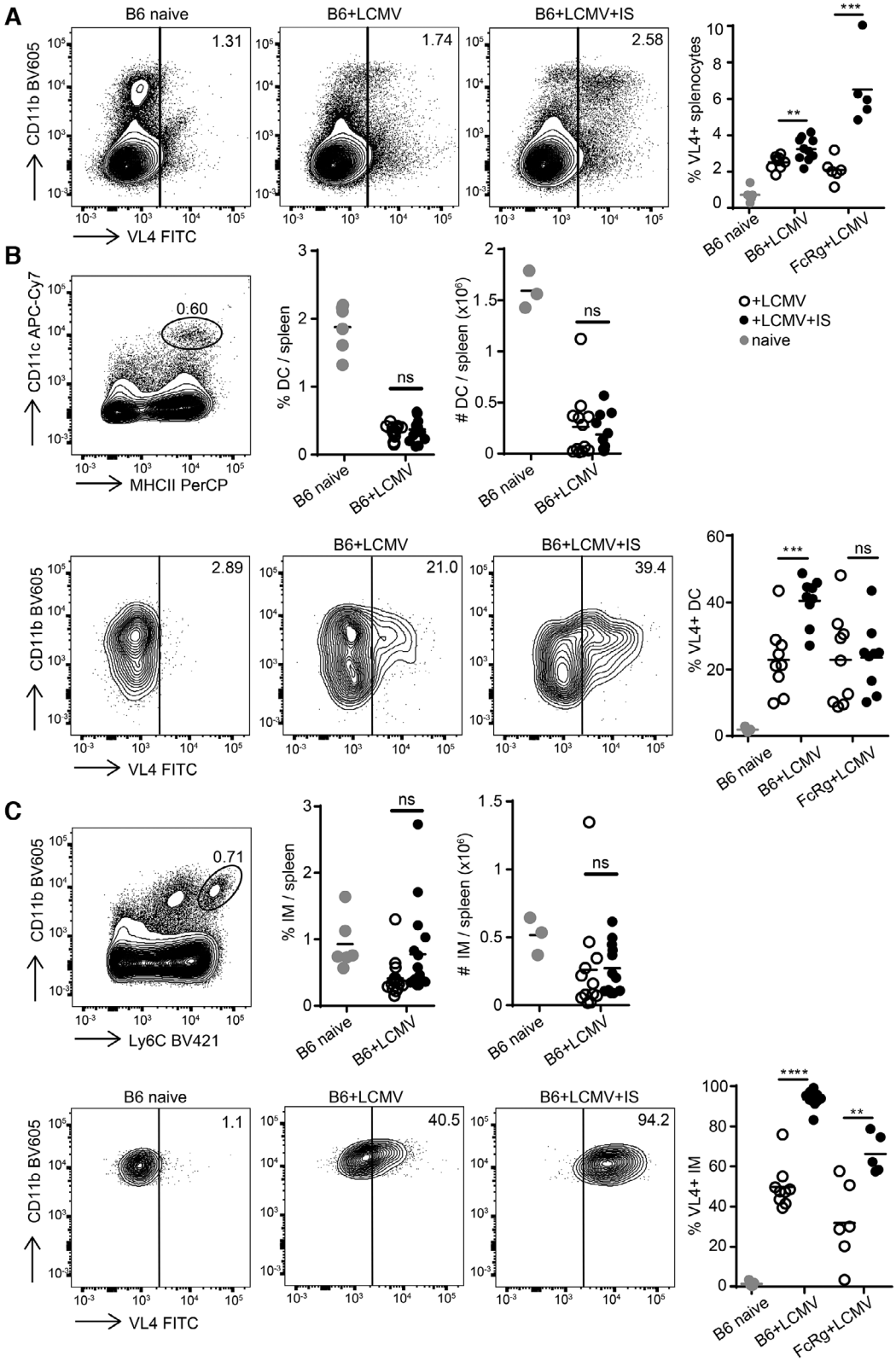

Figure 3. LCMV-specific nnAbs alter the tropism of virus-infected cells to IMs in an FcRγ-independent manner. WT and FcRγ-KO (FcRg) mice were infected with LCMV or LCMV opsonized with IS. Splenocytes were analyzed 3dpi by flow cytometry. Representative dot blots and histograms of $VL4^+$ single live (A) total splenocytes, (B) $CD11c^+MHCII^{hi}$ dendritic cells, and (C) $CD11b^+Ly6C^{hi}$ inflammatory monocytes are presented. Data are representative or pooled of two to four independent experiments with n = 3 per group. Horizontal line represents the mean. Statistical analysis was performed using two-tailed unpaired Student's *t*-test, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, ns = not significant.

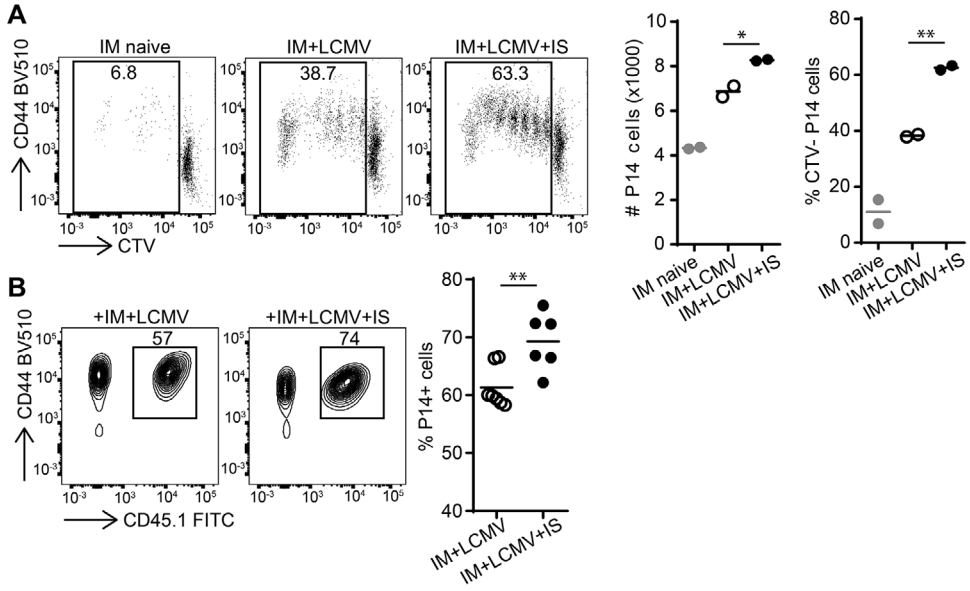

Figure 4. nnAb-primed IM from LCMV-infected mice enhance virus-specific T-cell proliferation. (A) IMs from WT mice infected with LCMV opsonized with or without IS for 3 days were sorted by FACS,gated on single live Ly6G$^-$CD11b$^+$Ly6C^{hi} cells. Sorted IMs were cocultured with CTV-labeled P14 cells and sorted endogenous CD11c$^+$MHCII^{hi} DCs from LCMV-infected mice for 3 days. Shown are representative dot blots of P14 cells, percentages of CTV-negative P14 cells, and calculated numbers of recovered P14 cells of two independent experiments performed in duplicates. (B) IMs from WT mice infected with LCMV with or without IS for 3 days were sorted by FACS, gated on single live Ly6G$^-$CD11b$^+$Ly6C^{hi} cells. Sorted IMs were retransferred in IM-deficient CCR2$^{-/-}$ mice together with naïve P14 cells and mice were infected with LCMV. Shown are representative blots and percentages of P14 cells gated on CD8$^+$ single cells in the blood of recipient mice 7dpi. Data of two independent experiments were pooled with n = 3 mice per group. Horizontal line represents the mean. Statistical analysis was performed using two-tailed unpaired Student's *t*-test, $^*p < 0.05$, $^{**}p < 0.01$.

were significantly increased when mice were infected and treated with IS (Fig. 5A and B) or KL53 (Supporting information Fig. S4C). Also, adoptively transferred P14 cells expanded significantly more in presence of IS than in its absence (Fig. 5C). Of note, this was also the case in FcR$\gamma^{-/-}$ hosts (Supporting information Fig. S4B), consistent with an FcRγ-independent mechanism by which LCMV-specific nnAbs enhance CD8$^+$ T-cell responses and prevent LCMV chronicity.

We also noted phenotypic differences in LCMV-specific CD8$^+$ T cells elicited in presence or absence of LCMV-specific nnAbs. In IS-treated mice, a higher proportion of total CD44^{hi} CD8$^+$ T cells, endogenous gp$_{33}$-specific as well as transgenic P14 T cells exhibited a phenotype compatible with short-lived effector cells (CD127$^-$ KLRG1$^+$) (Fig. 5D-F). Similar results were obtained when using KL53 instead of IS (Supporting information Fig. S4C). Furthermore, IS treatment decreased PD1 expression levels on P14 cells and increased the numbers of degranulating (CD107a$^+$) and IFNγ^+ P14 T cells (Fig. 5G).

Finally and most importantly, we measured the in vivo killing activity of LCMV-specific CD8$^+$ T cells raised in presence or absence of IS. For this, we infected mice with LCMV opsonized with or without IS. Seven dpi we transferred congenically marked splenocytes (CD45.1 and CD90.1) pulsed with different concentrations (10^{-7}, 10^{-8}, and 10^{-9} M) of gp$_{33}$ and np$_{396}$ peptide. Three hours later, we determined the specific killing of target cells in spleen and blood. Transfer of target cells into naïve B6 mice served as control. Both groups of infected mice were able to kill gp$_{33}$- and np$_{396}$-loaded target cells. However, mice that had received IS at the time of infection exhibited a significantly stronger killing capacity of all target cell populations both in spleen (Fig. 5H) and blood (Supporting information Fig. S4D), in support of a more potent LCMV-specific CD8$^+$ T-cell response induced in presence of LCMV-specific nnAbs.

Discussion

In contrast to virus-neutralizing antibodies, virus-specific nnAbs have not been extensively studied in the context of antiviral therapies, despite their broad range of physiological effects.

Deposition of nnAbs on virus particles or infected cells and subsequent recognition by FcRγ^+ cells was demonstrated for various viruses [1]. However, FcRγ-dependent recognition of nnAb-opsonized virus was associated with enhanced overall virus replication [24]. This so-called antibody-dependent enhancement (ADE) of viral infection is the major cause of fatal immunopathology of heterologous reinfection with Dengue virus [25]. This is one reason why nnAbs caught less attention in the context of antiviral therapies. However, nnAbs emerge as a potent tool to complement current HIV treatment, as nnAb activities interfering with HIV replication can also lead to a decrease in viral load and even in vivo protection [26].

The currently described protective mechanisms of nnAbs involve FcR on target cells and include antibody-dependent

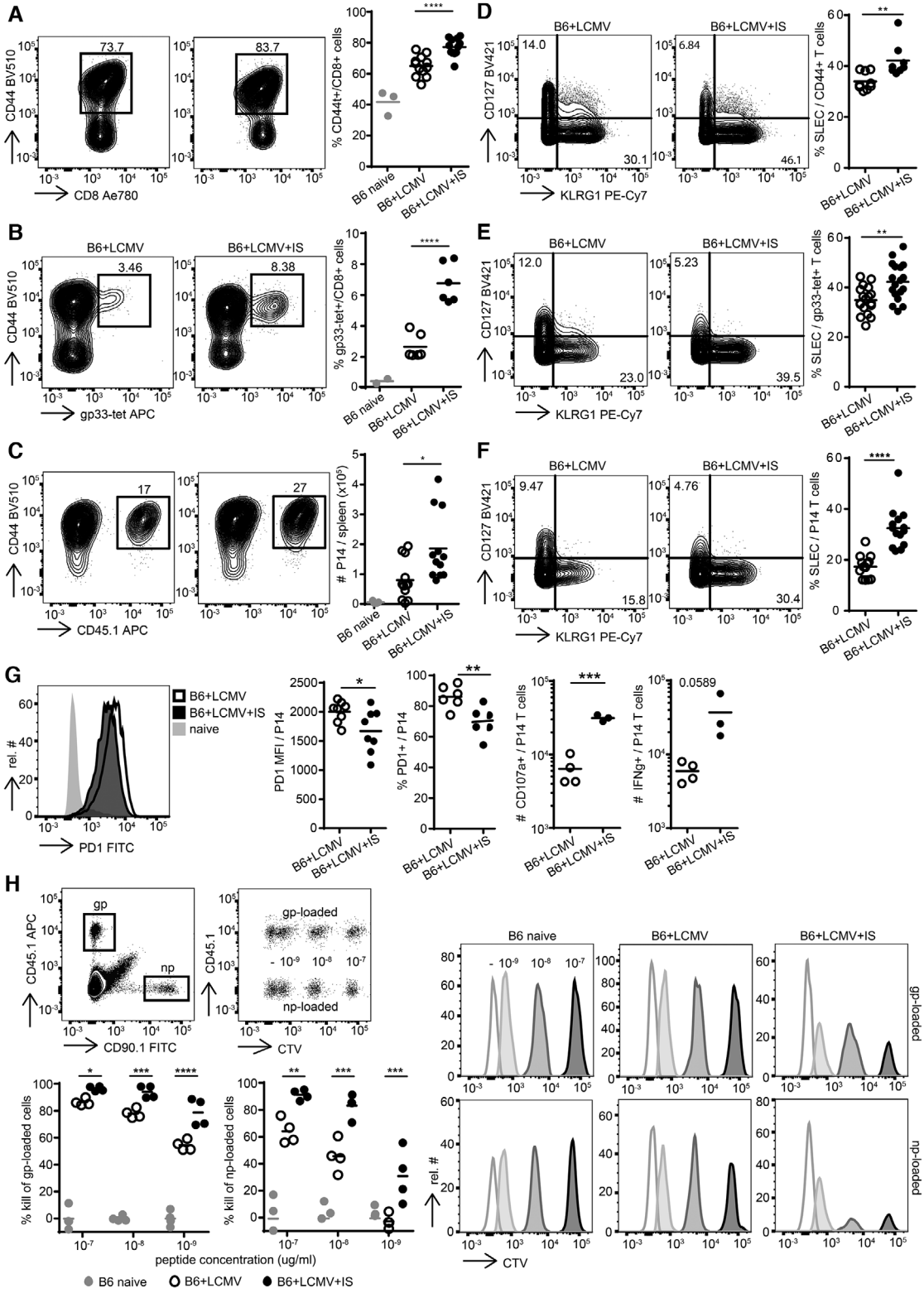

Figure 5. LCMV-specific nnAb increase virus-specific T-cell responses. (A, B, D, E) WT mice were infected with LCMV opsonized with or without IS. (C, F, G) WT mice received purified 10^4 P14 T cells 1 day prior infection with LCMV with or without IS. Splenocytes were analyzed 7dpi by flow cytometry. Data are representative of two to four independent experiments with n = 3-4 per group. (H) WT CD45.2 CD90.2 mice were infected with LCMV with or without IS. Seven dpi mice received a mixture of CTV-labeled CD90.1$^+$ np_{396}- or CD45.1$^+$ gp_{33}-loaded splenocytes. The percentage of killed target cells in infected mice was evaluated 3 h after transfer normalized to the percentage of target cells in naïve mice. Shown are representative data from two independent experiments with n = 3-4 per group. Statistical analysis was performed using two-tailed unpaired Student's *t*-test, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$, ns = not significant.

cellular cytotoxicity (ADCC), ADCP, complement-dependent cytolysis (CDC), or steric inhibition of viral proteins important for viral replication, assembly, and release [1, 2]. Richter and Straub et al. showed for the first time that LCMV-specific nnAbs protect against chronic infection in the absence of complement or activating Fc-receptors [8, 9]. A few years later, Duhan et al. showed that the presence of virus-specific antibodies redirect early LCMV infection from peripheral organs to the marginal zone of the spleen. This allowed boosting of the immune response and protection against chronic infection [19]. However, the enhanced infection of MMf by LCMV-specific antibodies was still FcR-dependent (Supporting information Fig. S3). Here, we include a new role of Ly6C^{hi} IMs in recognizing antibody-opsonized virus in an FcR-independent manner and participating in the protection against chronic LCMV infection by acting as additional APCs.

Previous studies have described a role of pre-existing humoral immunity in shaping the size and quality of antiviral T- cell responses in the context of chronic infection [4, 19, 27]. One way how pre-existing nnAbs were shown to promote T-cell immunity was by ADCP through FcR^{+} cells, leading to accumulation of antigen in secondary lymphoid organs [19, 27]. In the context of Dengue infection, CD8^{+} T cells were crucial in preventing ADE against heterotypic, but not homotypic reinfection in the presence of nnAbs [28]. The importance of the cooperativity of CD8^{+} T cells, nnAbs, and alveolar macrophages has been demonstrated in heterotypic influenza infection [4]. Laidlaw et al. showed that while virus-specific CD8^{+} T cells and virus-specific nnAbs alone were relatively ineffective, they elicited synergistic effects of protective immunity dependent on lung phagocytes.

In the context of chronic viral infections, virus-specific CD8^{+} T cells are often described as "exhausted," indicative of a differentiation status that is characterized by increased expression of coinhibitory receptors, such as PD1, Lag3, and Tim3, reduced effector functions like IFN-γ, TNF-α or IL2 secretion, or cytotoxicity [29]. Initially described for chronic LCMV infection, T-cell exhaustion was subsequently described in a wide range of human chronic diseases including chronic viral infection, autoimmunity, and cancer [30–32]. Numeric and functional reinvigoration of exhausted T cells bears significant opportunities for improving control of cancer and chronic infections [33]. However, such reinvigoration also bears the risk of (severe) immunopathology [34, 35]. Here, we show that preconditioning of mice with LCMV-specific nnAbs can prevent establishment of viral chronicity in the absence of overt immunopathology.

APCs play a decisive role in the control of chronic viral infections. LCMV infection disrupts DC function and homeostasis to facilitate persistent infection. Some of the described mechanisms include downregulation of MHC, costimulatory molecules, and proinflammatory cytokines, and the induction of immunosuppressive cytokines [36]. Here, we found that DC numbers and phenotype were not affected by nnAbs, although presence of nnAbs promoted DC infection in an FcR-dependent manner.

Conversely, we identified Ly6C^{hi} IMs being infected at markedly increased frequencies in presence of virus-specific nnAbs. In addition, these IMs acquired an activated, DC-like phenotype and served as additional APCs in T-cell priming. IMs are usually associated with detrimental effects in chronic diseases [37, 38]. Two particular studies describe the deletion of LCMV-specific B cells by Ly6C^{hi} IMs in response to Type 1 IFN [39, 40]. The treatment with nnAbs and the enhanced infection of Ly6C^{hi} IMs in our study was, however, not associated with changes in systemic Type 1 IFN levels (data not shown). A very recent study compared the transcriptional profile of Ly6C^{hi} monocytes from acute and chronic LCMV infection 6dpi [41]. While Ly6C^{hi} monocytes from acute infection expressed high levels of proinflammatory cytokines, chemokines, chemokine receptors, and transcription factors associated with M1 macrophages, monocytes isolated from chronically infected mice acquired an anti-inflammatory phenotype characterized by transcription factors and genes associated with M2 macrophage differentiation. While these data were obtained 6dpi, our data show that within the first day of chronic infection, virus-specific nnAb-treatment programed APC-like features in IMs, promoting early antigen presentation and T-cell priming.

The function of recruited monocytes is highly dependent on the type of infection, the tissue, and microenvironment [42]. Besides their detrimental effects in particular settings, monocytes have been shown to be crucial in transporting antigen to secondary lymphoid organs and thereby promoting T-cell activation in fungal [43], bacterial [44], and viral infections [45]. They can differentiate to proinflammatory DCs and prime Th1 responses [46]. Recently, it has been demonstrated in a murine *Mycobacterium tuberculosis* model that monocytes transport live bacteria to the draining LN which was required for efficient T-cell priming [44]. However, these monocytes failed to present antigens but instead transferred antigen to DCs in the draining LNs, which in turn primed naïve antigen-specific T cells [44]. A similar observation was published in the context of dermal fungal infection [43].

Monocytes can contribute to viral clearance or exacerbate pathological damage depending on the context of the infection [37]. Exploring the involvement of these cells in antiviral protection and pathology has the potential to create more effective antiviral treatments. Here, we show that in presence of LCMV-specific nnAbs, IMs were very effectively infected and served as additional APCs in early priming of the antiviral CD8 T-cell response, providing enough additional signal to generate higher frequencies of cytotoxic CD8^{+} T cells with lower PD1 expression, higher IFN-γ production, and higher in vivo killing ability, together preventing establishment of chronic infection without causing fatal immunopathology. We show that the nnAb-mediated protection against the establishment of chronic LCMV infection involves markedly enhanced FcRγ-independent infection of Ly6C^{hi} monocytes. The molecular mechanism of how Ly6C^{hi} monocytes are increasingly infected by opsonized LCMV in an FcRγ-independent manner remains to be solved. These findings emphasize the importance of nnAbs in preventing establishment of chronic viral infection and present a new role of IMs combining both humoral and cellular immunity.

Material and methods

Mice and virus

Mice were kept under specific pathogen-free conditions and animal experiments were performed according to the guidelines of the animal experimentation law (SR 455.163; TVV) of the Swiss Federal Government. The protocol was approved by the Cantonal Veterinary Office (animal experimentation number 127/2011 and 117/2017). C57BL/6J (B6; Janvier Elevage, France), FcR$\gamma^{-/-}$ mice (deficient for the Fc receptor gamma chain shared by all activating FcγRs), congenic CD45.1, Thy1a.Igha/J, CCR2$^{-/-}$ (deficient for circulating monocytes, kindly provided by Burkhard Becher), and gp33-specific TCR transgenic P14 mice expressing the congenic marker CD45.1 were bred and kept under at the ETH EPIC facility.

LCMV clone 13 was propagated on baby hamster kidney 21 cells, and viral titers were determined as described previously [47]. Mice were infected with 1-2.10^6 ffu i.v.

Antibodies and immune serum

LCMV NP-specific mAb was derived from mouse IgG2a secreting hybridoma KL53 [48] and purified by protein G sepharose chromatography (GE Healthcare, UK). IS was generated by infecting naïve B6 with 500 ffu of LCMV clone 13 for 12 days. If not explicitly stated otherwise, 450 μL IS, 0.5 mg KL53 or control IgG2a (BioXCell) were preincubated with 1-2.10^6 ffu LCMV for 20 min at room temperature and administered i.v. into the mice.

Preparation of single-cell suspension

Splenocyte suspensions were prepared by passing the spleens through a metal mesh using syringe plungers or by digesting them in RPMI containing 1 mg/mL Liberase (SigmaAldrich, Switzerland) and 0.2 mg/mL DNase I (Roche Diagnostics, Switzerland).

Purification of CD8$^+$ P14 T cells

Splenic CD8$^+$ T cells were isolated from CD45.1$^+$ P14 mice by immunomagnetic sorting according to the manufacturer's instructions (Miltenyi Biotec, Germany) and cultured either in vitro 5.10^4 cells/mL or transferred i.v. 1.10^4 cells/mouse into recipients 1 day prior to virus infection.

Flow cytometry

The antibodies used for flow cytometry are listed in Supporting information Table S1. All antibodies were purchased from BioLegend, eBioscience, and BD Biosciences. Surface stainings were performed at 4°C for 30 min in FACS-buffer supplemented with Fc-blocking antibody from 2.4G2 hybridoma. For the analysis of cytokine secretion and degranulation splenocytes were stimulated with 1 μg/mL gp33 peptide in the presence of Brefeldin A (10 μg/mL; Sigma) or Monensin A (2 μM; Sigma) and in the presence of anti-CD107 antibodies for 6 h at 37°C. For intracellular staining, cells were fixed with fixation buffer (BioLegend). Samples were measured with BD FACSCantoII, BD LSRFortessa, or BD FACSymphony (BD Biosciences) and analyzed with FlowJo software (Tree Star). For data analysis, we have adhered to the flow cytometry guidelines [49] and the gating strategy is depicted in Supporting information Fig. S5.

Retransfer of monocytes

IMs were defined as CD11b$^+$Ly6C$^+$Ly6G$^-$ and DCs as CD11c^{hi}MHC-II^{hi}. For sorting IMs and DCs, spleens were digested with Liberase and DNase I, depleted of CD19$^+$ cells using MojoSort nanobeads (BioLegend), stained for CD11b, CD11c, Ly6C, IAb, and Ly6G and sorted by FACS Aria (BD Biosciences). Sorted IMs were cocultured with MACS-purified P14 T cells and sorted DCs from LCMV-infected mice for 3 days in ratio IM:DC:P14 10:1:1.

In vivo cytotoxicity assay

For analysis of CTL activity of LCMV-infected mice, we transferred LCMV-peptide loaded splenocytes into B6 mice infected with LCMV for 7 days. We used splenocytes from CD45.1$^+$ mice loaded with 10^{-7}, 10^{-8}, 10^{-9} M of gp33-peptide and labeled them with 1, 0.5, and 0.1 μM of CellTrace Violet (CTV; ThermoFischer), respectively. Additionally, we used splenocytes from Thy1.1$^+$ mice loaded with 10^{-7}, 10^{-8}, 10^{-9} M of np396-peptide and labeled them again with 1, 0.5, and 0.1 μM of CTV, respectively. We transferred a mixture of unloaded and unlabeled and gp33- or np396-loaded CTV-labeled CD45.1$^+$ and Thy1.1$^+$ splenocytes into naïve or infected mice and calculated the cytotoxic abilities of the recipient mice according to the recovered target cells 3 h after transfer.

Cluster analysis

Dimensionality reduction

Cell populations were pregated (single live, CD45+, Lin- (CD3, CD19, NK1.1, Ly6G)) for DCs or IMs. Afterwards scaled marker expression (VL4, MHCII, CD209, CD24, CD11b, CD64, Ly6G, CD169, XCR1, Ly6C, CD40, CX3CR1, F480, CD11c) was used to compute tSNE (perplexity = 50) [50] in R.

Clustering

Cells gated on IMs from infected mice (treated or untreated with KL53 1dpi) were pooled and clustered based on their marker profile (VL4, MHCII, D209, CD24, CD11b, CD64, Ly6G, CD8,

CD169, XCR1, Ly6C, CD40, CX3CR1, F480, CD11c) using FlowSOM [51].

Heatmaps

Values of marker medians (or frequency of KL53$^+$ cells) were used to compute heatmaps in R with MADE4 package [52].

Statistical analysis

The two-tailed unpaired Student's *t*-test was applied for statistical analysis when the distribution was Gaussian. Otherwise, the Mann–Whitney test was performed. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; n.s. not significant.

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Abbreviations: **ADCP**: antibody-dependent cellular phagocytosi · **dpi**: days postinfection · **LCMV**: Lymphocytic choriomeningitis virus · **MMf**: metallophilic macrophages · **nnAbs**: non-neutralizing antibodies · **IS**: immune serum · **IM**: inflammatory monocyte · **FcRγ**: Fc gamma receptor

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