Fibroblastic reticular cells of the lymph node are required for retention of resting but not activated CD8$^+$ T cells

Alice E. Denton[a,1], Edward W. Roberts[a], Michelle A. Linterman[b], and Douglas T. Fearon[a,1]

[a]Department of Medicine and Cancer Research UK Cambridge Institute, University of Cambridge, Cambridge CB2 0RE, United Kingdom; and [b]Lymphocyte Signalling and Development, Babraham Institute, Cambridge CB22 3AT, United Kingdom

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Fibroblastic reticular cells (FRCs), through their expression of CC chemokine ligand (CCL)19 and CCL21, attract and retain T cells in lymph nodes (LNs), but whether this function applies to both resting and activated T cells has not been examined. Here we describe a model for conditionally depleting FRCs from LNs based on their expression of the diphtheria toxin receptor (DTR) directed by the gene encoding fibroblast activation protein-α (FAP). As expected, depleting FAP$^+$ FRCs causes the loss of naïve T cells, B cells, and dendritic cells from LNs, and this loss decreases the magnitude of the B- and T-cell responses to a subsequent infection with influenza A virus. In contrast, depleting FAP$^+$ FRCs during an ongoing influenza infection does not diminish the number or continued response of activated T and B cells in the draining LNs, despite still resulting in the loss of naïve T cells. Therefore, different rules govern the LN trafficking of resting and activated T cells; once a T cell is engaged in antigen-specific clonal expansion, its retention no longer depends on FRCs or their chemokines, CCL19 and CCL21. Our findings suggest that activated T cells remain in the LN because they down-regulate the expression of the sphingosine-1 phosphate receptor-1, which mediates the exit of lymphocytes from secondary lymphoid organs. Therefore, LN retention of naïve lymphocytes and the initiation of an immune response depend on FRCs, but is an FRC independent and possibly cell-autonomous response of activated T cells, which allows the magnitude of clonal expansion to determine LN egress.

viral infection | T-cell migration | lymph node stroma

The generation of optimal T-cell responses to infection is critically dependent on both clonal expansion and differentiation of T cells in reactive lymph nodes (LNs). Lymphocyte entry into LNs from the blood occurs at high endothelial venules and is chiefly mediated by interactions between CC chemokine receptor (CCR)7 on the T-cell, and CCL19 and CCL21 expressed by the specialized stromal component, the fibroblastic reticular cell (FRC) (1–3). Lymphocyte egress from LNs occurs at lymphatic sinuses and requires the sphingosine-1 phosphate receptor-1 (S1PR1) (4, 5). Previous studies have demonstrated that S1PR1, at least in part, operates to overcome the CCR7-mediated retention signal to allow egress of naïve T cells into the lymph (5, 6), and it has been suggested that a similar balance of signals controls the egress of activated T cells.

Whereas the role of FRCs in the regulation of resting naïve T-cell migration and localization is inferred based on their biosynthesis of CCL19 and CCL21, the impaired LN homing of CCR7-deficient lymphocytes, and the defects in LN homing and homeostasis observed in CCL19/21-deficient and CCL19-knockout mice (2, 3, 7), in vivo proof of this function is lacking. Moreover, our understanding of whether FRCs similarly regulate the retention in the LNs of activated T cells is even less complete. That FRCs participate in an active immune response is suggested by the transcriptional changes that they exhibit in response to inflammation (8), the role of stromal-derived retinoic acid in promoting expression of gut-homing integrins by T cells (9), and by the proposal that their "maturation" may be required for the generation of antiviral immune responses (10). Recent studies have also suggested that LN FRCs suppress T-cell proliferation through their expression of Nos2 in response to T-cell–derived interferon (IFN)-γ (11–13). Thus, whereas a variety of studies have pointed toward a significant involvement of FRCs in the generation of immune responses, there has not been an appropriate in vivo model in which to test these proposed biological roles of the FRC.

Fibroblast activation protein-α (FAP) is a marker of stromal cells that was originally described in human adenocarcinomas and at sites of wound healing and subsequently was demonstrated also at sites of chronic inflammation and immune privilege (14–17). Whereas FAP$^{-/-}$ mice are phenotypically indistinct from wild-type mice (18), the stromal cell that expresses FAP mediates immune suppression in the tumor microenvironment (19, 20) and maintains skeletal muscle mass and hematopoiesis (21, 22). FAP$^+$ stromal cells from different organs display similar gene expression profiles, but adopt organ-specific functions, like the production of specific cytokines and chemokines. For example, FAP$^+$ stromal cells in skeletal muscle maintain muscle mass through the production of follistatin, whereas bone marrow (BM)-derived FAP$^+$ cells include CXC chemokine ligand (CXCL)12-abundant reticular (CAR) cells and support hematopoiesis by producing CXCL12, interleukin (IL)-7, and kit-ligand (21, 23). The roles of FAP$^+$ stromal cells in other tissues and organs have not yet been defined.

In this study we demonstrate that LN FRCs express FAP and describe for the first time to our knowledge a model in which LN

Significance

The balance between the retention of lymphocytes in lymph nodes and their exit is a key factor determining clonal burst size, differentiation, and the efficacy of pathogen clearance. Currently, it is understood that naïve and activated T cells regulate their migration using a similar system, that is, balancing CC chemokine receptor (CCR)7-mediated retention signals against Sphingosine-1 phosphate receptor-1 (S1PR1)-mediated exit signals. This study utilizes a mouse model to selectively deplete the cellular source of CCR7 ligands, the fibroblastic reticular cells, before or during viral infection. The study concludes that while retention of naïve lymphocytes does depend on the fibroblastic reticular cell, it may be a cell-autonomous response of activated T cells that allows them to determine in a cell-intrinsic manner the magnitude of clonal expansion.

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[1]To whom correspondence may be addressed. Email: dtf1000@cam.ac.uk or ad596@cam.ac.uk.

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FRCs, and thus their biosynthetic products, can be conditionally depleted from intact adult LNs. This model permits the demonstration that FRCs regulate the trafficking of resting lymphocytes, as was anticipated, but not that of activated lymphocytes, showing for the first time to our knowledge that the state of the lymphocyte dictates the rules governing its egress from LNs.

Results

LN FRCs Are Marked by FAP Expression and Mediate Normal LN Homeostasis. We previously described a bacterial artificial chromosome (BAC) transgenic (Tg) mouse line (DM2) (21) in which the *fap* gene in the BAC has been modified by the insertion of a cassette composed of the coding sequences of the primate diphtheria toxin (DTX) receptor (DTR) and firefly luciferase. In this Tg mouse, *fap*-driven luciferase expression was detected in the inguinal LN (iLN) but not the spleen, consistent with the relative levels of *Fap* mRNA in these tissues (Fig. 1*A*). Among enzymatically dispersed CD45$^-$ stromal cells from iLNs stained for FAP and the typical LN stromal markers, CD31 and gp38 (podoplanin), almost all FAP$^+$ cells were CD31$^-$gp38$^+$, which identifies them as FRCs (Fig. 1*B*). Within the four subsets of LN stroma defined by these markers, FAP was detected on >95% of CD31$^-$gp38$^+$ FRCs and 10–15% of CD31$^-$gp38$^-$ double-negative cells (DNCs), and not on blood endothelial cells (BECs; CD31$^+$gp38$^-$) or lymphatic endothelial cells (LECs; CD31$^+$gp38$^+$) (Fig. 1*C*). This flow cytometric analysis correlated with the distribution of *Fap* mRNA among the sort-purified FRC and DNC populations (Fig. 1*D*). The CD45$^+$FAP$^+$ population, which comprises a subset of F4/80^{hi}heme oxygenase-1(HO-1)$^+$ macrophages (24), were not found in the lymph node (Fig. S1); therefore, FAP expression is restricted to CD45$^-$ cells in this tissue. FAP$^+$ stromal cells also were the major source of the cytokines and chemokines reported to be associated with FRCs, including *Il7*, *Ccl19*, and *Ccl21* (Fig. 1 *E* and *F*). Confocal microscopy confirmed the localization of FAP$^+$ stromal cells to the T-cell zone of the LN, where FAP immunostaining colocalized with that of gp38 (Fig. 1*G*). FAP staining rarely colocalized with the follicular dendritic cell (FDC) marker, CD35 (Fig. 1*H*).

These findings demonstrated that FAP marks the FRCs of the LN stroma, thus allowing a direct rather than inferential analysis of the roles of these cells by conditionally depleting them from intact adult LNs. DM2 BAC Tg mice and littermate (Lm) controls were given DTX on 3 consecutive days and the constituent cells of the LN were determined 1 d later. DTX depleted FAP$^+$ and gp38$^+$ FRCs, with numbers of BECs, LECs, and DNCs being unchanged from those of the littermate controls (Fig. 2*A*). There was a significant decrease in B cells, T cells (both CD4$^+$ and CD8$^+$), natural killer (NK) cells, dendritic cells (DCs) and CD11b$^+$ cells (Fig. 2*B*). The spleens of DTX-treated DM2 mice did not differ from those of littermate controls (Fig. 2*B*), in accordance with the splenic FRC being FAP$^-$ and having a different origin from that of LN FRCs (25). Depletion of the FAP$^+$ stromal cells resulted in substantial disruption of the gp38$^+$ FRC network throughout the T-cell zone within 1 d of the last DTX treatment, with both the extracellular matrix, as defined by laminin staining, and the localization of the remaining B cells to the follicle being maintained (Fig. 2*C*). FDCs were readily detectable by confocal microscopy in FAP$^+$ cell-depleted LNs (Fig. 2*C*), consistent with their not expressing FAP (Fig. 1*H*) and the maintenance of B-cell follicle structure (Fig. 2*C*). Because FAP marks only ~15% of the gp38$^-$CD31$^-$ DNCs (Fig. 1*C*), their depletion was masked in this instance, but a similar analysis of LNs 10 d after DTX treatment did show a significant decrease in DNCs (Fig. S2*A*). The loss of FAP$^+$ stromal cells, lymphocytes, and DCs from the DM2 LN was maintained for at least 10 d after DTX treatment, and there was a slightly greater effect on the conventional DCs (cDCs) relative to migratory DCs (migDCs) (Fig. S2*B*). Moreover, the extracellular laminin network of the T-cell zone was maintained 10 d after FAP depletion, albeit with a condensed structure most likely due to LN shrinkage secondary to the loss of lymphocytes (Fig. 2*C*). Adoptive transfer of CFSE-labeled CD8$^+$ T cells into mice depleted of FAP$^+$ stromal cells showed deficient homing to inguinal (i) and mesenteric (mes) LNs, and Peyer's patches, but comparable numbers of transferred cells in the blood and spleen (Fig. 2 *D*), providing additional evidence that LN FAP$^+$ FRCs mediate lymphocyte immigration to LNs and Peyer's patches. This role of the FAP$^+$ FRC is likely explained by these cells being the major source of *Il7*, *Ccl19*, and *Ccl21* mRNA (Figs. 1*F* and 2*E*). Accordingly, depleting FAP$^+$ FRCs resulted in loss of CCL21 protein from the T-cell zone of the LN (Fig. 2*C*). The modest reduction in *Cxcl13* mRNA is likely to be due to depletion of FRCs that

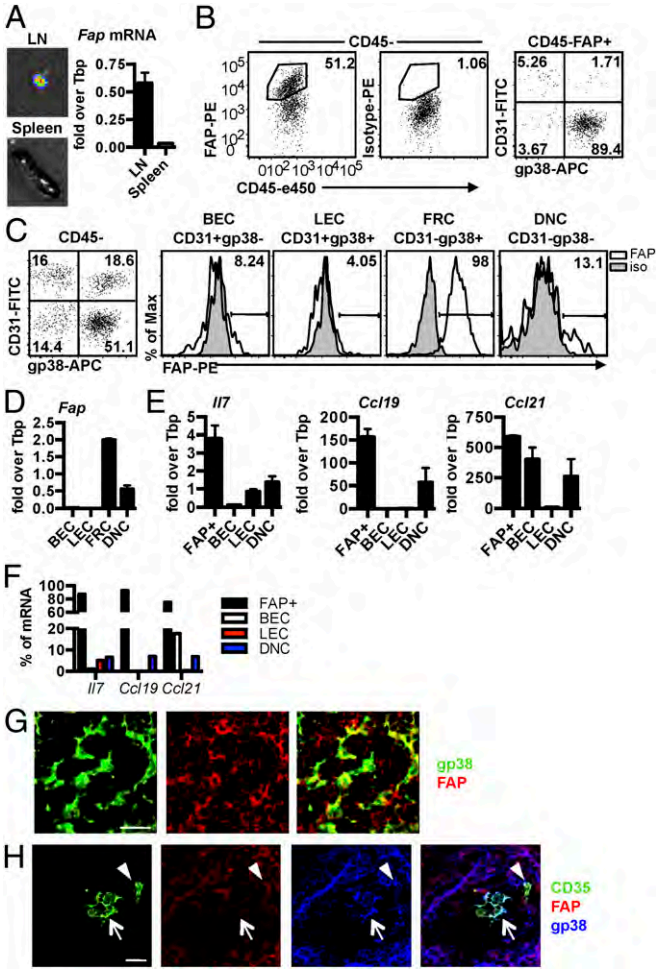

Fig. 1. Characterization of FAP-expressing FRCs from the LN. (*A*) Presence of FAP BAC Tg-driven luciferase activity in the iLN and absence in spleen was shown by bioluminescence, and *Fap* mRNA levels (relative to those of *Tbp*) in the iLN and spleen were determined by quantitative RT-PCR (qRT-PCR). Data are representative of two independent experiments and represented as mean + SEM (n = 5). (*B* and *C*) The expression of FAP, CD31, and gp38 on CD45- cells of enzymatically dispersed, single cell suspensions from the iLN was determined by flow cytometry. (*D*) The levels of FAP mRNA in sort-purified LN stromal cell populations were measured by qRT-PCR. Data are the average of two sorts with each sort being composed of LNs pooled from three to five mice and are expressed as mean + SEM. (*E*) The levels of cytokine and chemokine mRNAs in sort-purified LN FAP$^+$ cells, BECs, LECs, and DNCs were measured by qRT-PCR. Data are the average of two to three sorts with each being composed of LNs pooled from three to five mice and are expressed as mean + SEM. (*F*) The relative mRNA levels for *Il7*, *Ccl19*, and *Ccl21* for each stromal cell type were determined by qRT-PCR. (*G* and *H*) Localization of FAP staining in LN sections, showing extensive colocalization with gp38 on FRCs in the T-cell zone (*G*), and rare colocalization with CD35 on FDCs in the follicular zone (*H*). FAP$^+$ FDC (arrowhead) and FAP$^-$ FDC (arrow) are indicated (*H*). (Scale bars, 20 μm.)

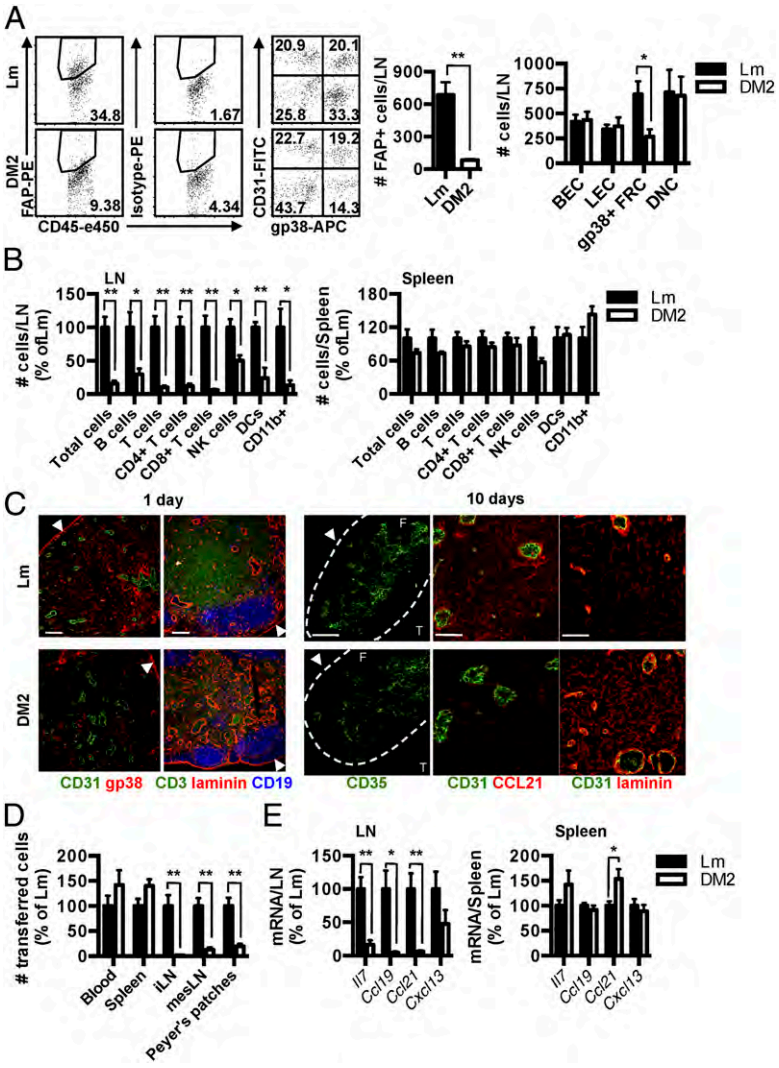

Fig. 2. Conditionally depleting FAP$^+$ cells disrupts normal LN homeostasis. (*A* and *B*) Littermate (Lm) and DM2 BAC transgenic mice were given DTX on 3 consecutive days. The stromal cell types in the LN were then measured by flow cytometry (*A*) as were the lymphocyte and DC populations in the LN and spleen (*B*), 1 d after the final DTX dose. (*C*) Representative immunofluorescence staining of LNs derived from Lm and DM2 mice, showing the gp38$^+$ FRC network in the T-cell zone and B- and T-cell staining with the extracellular laminin network in mice treated as in *A* and *B*, or 10 d after DTX administration showing CD35$^+$ FDCs in the follicle and CCL21 protein and the extracellular laminin network in the T-cell zone. Arrowheads indicate the LN capsule/edge, dotted lines identify regions of the LN: F, follicle and T, T-cell zone. [Scale bars, 100 μm (1 d) or 50 μm (10 d).] (*D*) The homing of adoptively transferred CD8$^+$ T cells to various lymphoid compartments was determined 10 d after FAP$^+$ FRC depletion. (*E*) At the same time point following DTX administration as in *A* and *B*, mRNA copies of *Il7*, *Ccl19*, *Ccl21*, and *Cxcl13* were measured in the LN and spleen. Data are representative of three independent experiments ($n = 4$), and, where appropriate, expressed as mean + SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

express *Cxcl13* (8, 26) and not the *Cxcl13*-expressing FDCs (Fig. 2*C*).

Roles of the LN FAP$^+$ FRC in Viral Infection. To examine the contribution of FRCs to the development of T- and B-cell immune responses, we intranasally infected mice with influenza A virus. This results in an acute localized pulmonary infection that generates robust antiviral T- and B-cell responses in the mediastinal (med) LN. Moreover, because FAP$^+$ stromal cells are absent from the adult mouse lung (21), alterations in the infection process that may be associated with the depletion of FAP$^+$ stromal cells would be secondary to impaired responses in the draining LN and not to a direct effect on the lungs.

An increase in CD45$^+$ cells was the first change to occur in the medLNs, followed by increased numbers of FAP$^+$ FRCs, BECs, and LECs 2 d later (Fig. 3*A*). This expansion of the stromal cell populations and CD45$^+$ cells peaked 9 d postinfection, with all groups contracting thereafter except for LECs, which remained increased at day 30 postinfection. Influenza A virus infection 10 d after the DTX-mediated depletion of FAP$^+$ FRCs, a time at which LNs are still disrupted (Fig. S2 and Fig. 3*B*), caused a comparable fold increase in the LN stromal cell types, indicating that the residual FRCs maintained a capacity for responding to an acute infection. This response, however, did not compensate for the initial deficit caused by DTX treatment (Fig. 3*C*).

To assess the effects of specifically inducing a deficiency of FRCs, we measured various parameters of the antiviral immune response 6 d postinfection. The numbers of influenza-specific CD8$^+$ T cells in the medLNs, lung, and spleen were determined using a D^bPA_{224}-specific dextramer (CD8$^+$D^{b}PA$^+$) (Fig. 4*A*). The medLN was also assessed for the development of germinal center (GC) B cells (B220$^+$CD95$^+$Bcl6$^+$), plasma B cells (B220^{int}CD138$^+$), and T follicular helper (Tfh) cells (CD4$^+$Foxp3$^-$CXCR5$^+$PD1$^+$). Both the antiviral T- and B-cell responses were significantly lower in the mice previously depleted of FAP$^+$ stromal cells (Fig. 4*A*). We further characterized the abnormal B-cell response by administering DTX to littermate and DM2 mice and immunizing them with s.c. NP-KLH (4-Hydroxy-3-nitrophenylacetyl conjugated to Keyhole Limpet Hemocyanin) emulsified in alum. Ten days after immunization, the Tfh cell, NP-specific GC B-cell (defined as B220$^+$CD95$^+$ Ki67$^+$NP$^+$) and NP-specific plasma B-cell (B220^{int}CD138$^+$NP$^+$) responses were significantly diminished (Fig. 4*B*). These impaired responses resulted in a proportionate decrease in both low- and high-affinity NP-specific antibodies, indicating that the overall diminution of the humoral response occurred without affecting affinity maturation (Fig. 4*B*). Taken together, these findings demonstrate that reducing FAP$^+$ FRCs before infection disrupts LN homeostasis and impairs the generation of antiviral CD8$^+$ T cells, Tfh cells, and B cells.

Because LN FAP$^+$ FRCs are the main source of the CCR7 ligands, CCL19 and CCL21, the DM2 mouse model offers the

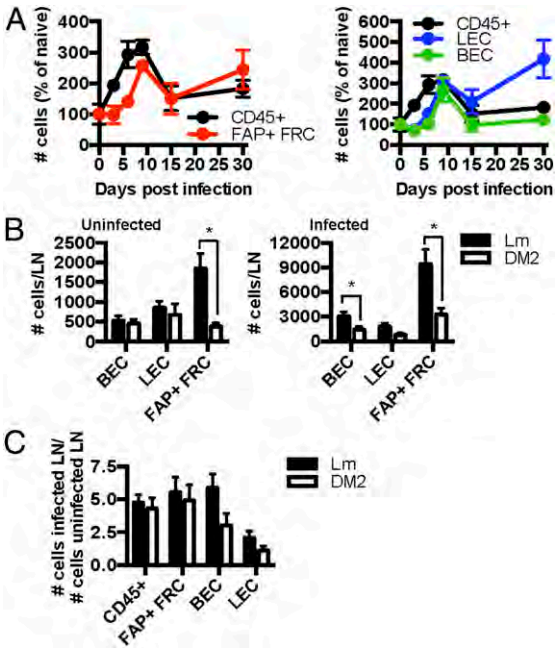

Fig. 3. Expansion of LN stromal cells during influenza A virus Infection. (*A*) B6 mice were infected with influenza A virus and the expansion of CD45$^+$ cells, FAP$^+$ FRCs, BECs, and LECs was determined by flow cytometry at different days postinfection. Data are representative of two independent experiments (n = 4–5), with results expressed as mean ± SEM (*B*) Littermate and DM2 mice were administered DTX on 2 consecutive days and 10 d later were infected with influenza A virus. Six days postinfection the number of stromal cells in the medLN of uninfected and infected mice was determined by flow cytometry. Data are representative of two independent experiments (n = 4–5), with results expressed as mean + SEM. *P < 0.05, **P < 0.01, ***P < 0.001. (*C*) The fold expansion of different cell subsets, relative to uninfected mice, was calculated from data shown in *B*.

opportunity to determine the contribution of CCR7-mediated retention signals to an ongoing antiviral immune response in vivo. Littermate and DM2 mice were infected with influenza A virus and DTX was administered on days 7–9 of infection (Fig. 5*A*) during the expansion and differentiation of CD8$^+$ T cells, GC B cells, and Tfh cells (27, 28). The medLNs, lung, and spleen were analyzed on day 10 for the CD8$^+$ T-cell responses; the Tfh, and B-cell responses were measured in the medLNs only. FAP$^+$ FRCs were diminished in the draining medLNs and nondraining iLNs, which resulted in decreased mRNA levels of *Il7*, *Ccl19*, and *Ccl21* (Fig. S3), and a marked diminution in the numbers of CD44^{lo} CD8$^+$ T cells (Fig. 5*A*). However, and in contrast to predictions of FRC-mediated immune suppression (11–13), there was no increase in the number of antigen-specific CD8$^+$ T cells in the medLNs, lung, or spleen (Fig. 5*B*) nor in the number of Tfh, GC B cells, or plasma cells (Fig. 5*C*). Moreover, we observed no change in the number of D^{b}PA-specific CD8$^+$ T cells when FAP$^+$ FRCs were depleted earlier in the antiviral response (Fig. S4). The depletion of FAP$^+$ cells also did not change the proportion of proliferating D^{b}PA-specific CD8$^+$ T cells (Fig. 6*A*). There was, however, a decrease in IL7Rα$^+$killer cell lectin-like receptor subfamily G member (KLRG)1$^-$ memory precursor effector cells (MPECs) and an increase in IL7Rα$^-$KLRG1$^+$ short lived effector cells (SLECs) in the medLNs (Fig. 6*B*), suggesting that depleting FAP$^+$ FRCs during the late phase of the immune response results in increased terminal differentiation of virus-specific CD8$^+$ T cells. This finding may reflect an increased frequency of contact between activated cells, secondary to the loss of naïve T cells, which triggers the Hippo pathway and induces the expression of Blimp-1 (29).

The differential response of naïve and activated CD8$^+$ T cells to FAP$^+$ FRC depletion suggested that activated CD8$^+$ T cells, in contrast to naïve cells, do not use CCR7-mediated signals for their retention in the LN. Considering that egress from LNs requires signaling through S1PR1 (4), we determined the expression of S1PR1 in CD8$^+$ T cells in the medLNs during influenza A virus infection. Activated CD8$^+$ T cells (either D^{b}PA-specific or CD44^{hi}CD62L^{lo}) have lower mRNA levels for *S1pr1* relative to that of naïve CD8$^+$ T cells from the medLN (Fig. 6*C*). Moreover, staining for surface S1PR1 expression suggests that D^{b}PA$^+$CD8$^+$ T cells in the medLN do not express S1PR1, whereas D^{b}PA$^+$CD8$^+$ T cells that have recirculated to the iLN have regained expression of this receptor (Fig. 6*D*). Taken together, these findings suggest that FRCs are required for naïve T-cell migration to and/or retention by responding LNs, but are dispensable for the retention of activated CD8$^+$ T cells. Rather, it is likely that the reacquisition of S1PR1 expression is the key signal for egress of activated T cells.

Discussion

LNs provide the appropriate microenvironment for the clonal expansion and differentiation of lymphocytes in response to microbial infections. FRCs have been considered to contribute to LN function by promoting the accumulation of naïve T cells and facilitating contacts between T cells and antigen-bearing DCs (1, 30). However, the role of FRCs with respect to the functions and trafficking of activated T cells has not been assessed in vivo because it has not been possible to alter experimentally these cells or their products after the onset of an infection. Naïve T cells transit through LNs relatively rapidly, residing for a mean of only 12–18 h (31); if activated T cells

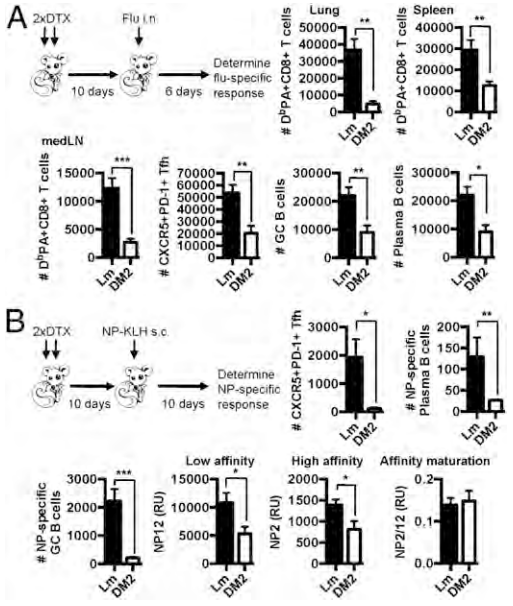

Fig. 4. Impaired antiviral T- and B-cell responses in LNs depleted of FAP$^+$ FRCs before infection. (*A*) Littermate and DM2 mice were given DTX on 2 consecutive days and infected with influenza A virus 10 d later. Six days postinfection, the D^{b}PA$^+$CD8$^+$ T-cell response was enumerated in the spleen, lung, and medLN by dextramer staining. The Tfh cell and GC and plasma B-cell responses were determined by antibody staining in the medLN only. Data are representative of three independent experiments (n = 5–6), with results expressed as mean + SEM. *P < 0.05, **P < 0.01, ***P < 0.001. (*B*) Littermate and DM2 mice that had been treated with DTX were immunized with NP-KLH 10 d later. The Tfh cell and NP-specific GC and plasma B-cell responses, as well as low- and high-affinity NP-specific IgG and the ratio between the two measurements, were determined 10 d after immunization. Data are representative of two independent experiments (n = 4), with results expressed as mean + SEM. *P < 0.05, **P < 0.01, ***P < 0.001.

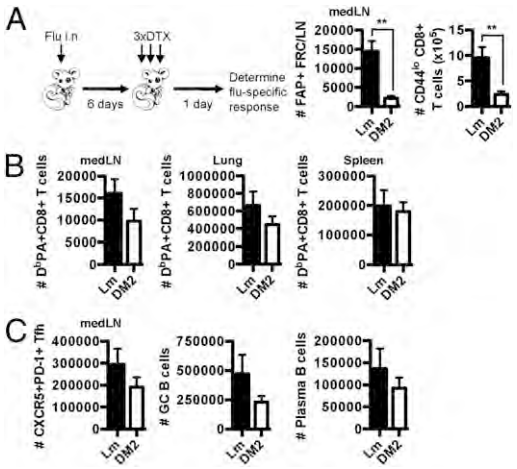

Fig. 5. Unimpaired antiviral T- and B-cell responses in LNs depleted of FAP$^+$ FRCs during the course of an acute viral infection. Littermate and DM2 mice were infected with influenza A virus and administered DTX on days 7–9 of infection. (*A*) The numbers of FAP$^+$ FRCs and CD44^{lo} CD8$^+$ T cells were measured in the medLN on d 10. (*B*) D^{b}PA-specific CD8$^+$ T cells were enumerated in the lung, spleen, and medLN by dextramer staining on day 10. (*C*) Tfh cell and GC and plasma B-cell responses were enumerated in the medLN by antibody staining on day 10. Data are representative of two independent experiments (n = 4–6), with results expressed as mean + SEM. $^{**}P < 0.01$.

obeyed the same rules as naïve T cells, clonal expansion and effector differentiation would be severely limited, leading to inefficient control of an infection. Therefore, different rules must regulate the trafficking of naïve and activated T cells.

Because FAP marks the FRCs of the LN, and because our laboratory has generated mouse lines in which the FAP-expressing cells can be conditionally depleted, we were able to directly assess FRC functions in resting and responding LNs. We found that FRCs are required for the maintenance of a normal resting LN architecture, and thus are indispensable for the initiation of T- and B-cell responses to viral infections. However, once an immune response is established, activated T and B cells no longer depend on the presence of FRCs; FRCs neither control the continued development nor suppress the proliferation of T and B cells in the late phase of a viral infection. Moreover, depletion of this cellular source of the CCR7 ligands, CCL19 and CCL21, does not result in increased release of activated CD8$^+$ T cells from the responding LN, suggesting that activated T cells are not retained in the LN by CCR7-mediated signals. Instead, activated CD8$^+$ T cells in responding LNs down-regulate the expression of S1PR1, the receptor that mediates lymphocyte egress from LNs. The detection of S1PR1 on antigen-specific CD8$^+$ T cells that have left the draining LN and circulated to a nondraining lymph node suggests that reacquisition of S1PR1 expression is the essential signal for LN egress of activated T cells.

Once activated by antigen-bearing DCs, naïve T cells enter a program of differentiation that leads to the generation of effector T cells that traffic to the site of infection (32), but the potential contributions of FRCs to different stages of the differentiation program of activated T cells have not been investigated. Previous studies have shown that mice lacking CCL19/21 (paucity of lymph node T cells or *plt*) display delayed but enhanced immune responses (7, 33, 34). Whereas there is an altered pro–Th1-type inflammatory infiltrate in immunized *plt* LNs (34), other studies suggest that T-cell responses in these mice may be generated in the splenic red pulp or the cortex of the LN (7), potentially contributing to the enhanced responses observed. We have demonstrated here that FAP$^+$ FRCs are critical to the initiation of T- and B-cell responses, as their depletion severely limits the pool of naïve cells in which are found antigen-specific cells. Whether the immune response is enhanced in the long term in this system is not known, and the limitations of this system of stromal cell depletion do not allow determination of the effect of FAP$^+$ FRC depletion on the immune response beyond 6 d of influenza A virus infection. However, given that our data demonstrate that depletion of FAP$^+$ FRCs during the immune response does not alter the continued development of T- or B-cell responses, it is likely that FAP$^+$ FRCs contribute to the generation of immune responses mainly by maintaining the pool size of naïve T and B cells in LNs.

During a T-cell response, the emigration of replicating T cells must be tightly controlled for an effective immune response. When T cells are activated, they must be retained in LNs until sufficient clonal expansion has been achieved. Activated T cells then terminally differentiate and acquire the capacity to leave the draining LN and traffic to the site of infection, where they control the pathogen. The migration patterns of naïve T cells are well defined. CCR7-mediated signaling is the essential determinant of T-cell immigration into LNs (2), whereas S1PR1 provides the exit signal (4). Whereas these two signals are balanced in naïve T cells (6), activated T cells down-regulate S1PR1 to increase their retention in LNs (4) and allow their continued proliferation. Whereas CCR7 expression can be down-regulated by TCR-mediated stimulation, it has also been shown that proliferating T cells maintain CCR7 expression during viral infection, with this receptor down-regulated only after multiple divisions (35), when terminal differentiation and LN egress would occur. This correlative evidence suggested that activated T

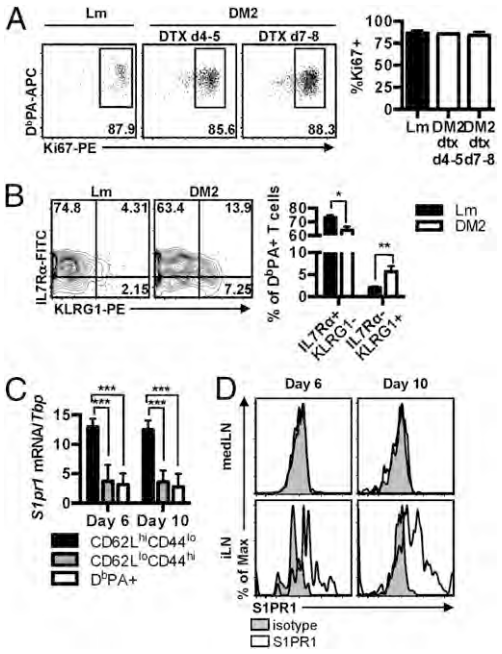

Fig. 6. Phenotypic characteristics of virus-specific CD8$^+$ T cells in the presence and absence of FAP$^+$ FRCs. (*A*) Lm and DM2 mice were infected with influenza A virus and administered DTX on either days 4–5 or 7–8 of infection, and the proliferation was measured on day 10, as assessed by Ki67 staining of D^{b}PA-specific CD8$^+$ T cells in medLN. Results are expressed as mean + SEM (n = 2–3). (*B*) Lm and DM2 mice were infected with influenza A virus and administered DTX on days 7–9 of infection and the expression of KLRG1 and IL7Rα by D^{b}PA-specific CD8$^+$ T cells in medLN was determined on day 10. Results are expressed as mean + SEM (n = 5) $^*P < 0.05$, $^{**}P < 0.01$. (*C*) Expression of *S1pr1* mRNA (relative to *Tbp*) was measured in sort-purified CD44^{lo}CD62L^{hi}, CD44^{hi}CD62L^{lo}, and D^{b}PA-specific CD8$^+$ T cells from the draining medLNs 6 d and 10 d after infection. Results are expressed as mean + SEM (n = 4–5). Statistical analysis was performed with one-way ANOVA $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (*D*) S1PR1 protein staining on D^{b}PA$^+$ CD8$^+$ T cells obtained from the medLN and iLN was determined 6 d and 10 d after infection. Data are representative of four mice.

cells were thought to balance the CCR7 retention and S1PR1 exit signals, as had been described for naïve T cells, and that both the loss of CCR7 and the reacquisition of S1PR1 must occur in order for activated T cells to egress from lymph nodes (6). However, by conditionally depleting the stromal cell source of the CCR7 ligands, we demonstrate that they, and by implication their receptor, CCR7, have no role in the retention of activated T cells in responding LNs. Our data do not preclude a role for other, non–CCR7-mediated, Gαi protein-coupled receptors in the retention of activated T cells (6), but do suggest that S1PR1 may play the dominant role in determining the migratory behavior of clonally expanding T cells. Consistent with this proposal is the finding that antigen-specific $CD8^+$ T cells in the draining LN do not express S1PR1, but those that have recirculated do. Because rapid egress from the responding LN limits the size of the effector pool (36, 37), this cell-autonomous behavior of activated T cells may be critical to the generation of T-cell responses of sufficient size to clear an infection. In conclusion, by using the first model in which FRCs can be conditionally depleted from intact adult LNs, we have confirmed the nonredundant role of FAP^+ FRCs in LN retention of naïve T cells and have made the unanticipated observation that clonally expanding T cells continue their developmental program in the absence of FRCs, demonstrating that different rules regulate the retention of naïve and activated T cells. For further discussion, see *SI Discussion*.

Materials and Methods

Mice. Male and female C57BL/6 (B6), DM2, and albino B6 mice at least 6 wk old were housed under specific pathogen-free conditions. For further details, see *SI Materials and Methods*.

Cell Isolation, Flow Cytometry, and Cell Sorting. Tissue samples were analyzed as described in *SI Materials and Methods*.

Immunofluorescence Microscopy. Slides were prepared and stained as specified in *SI Materials and Methods*.

Real-Time PCR. RNA was extracted and analyzed as described in *SI Materials and Methods*.

Infection, Immunization, and Determination of Serum IgG. For viral infection, mice were infected with 10^4 plaque-forming units of influenza A/HK/x31 virus (H3N2) i.n. under inhalation anesthesia with isoflurane. For antigen-specific B-cell responses, mice were immunized s.c. with NP-KLH (Biosearch Technologies) emulsified in alum (Thermo Scientific) and serum NP-specific IgG (NP2 and NP12) determined as previously described (38).

Statistical Analyses. Statistical analyses were conducted using an unpaired two-tailed (Student's) *t* test, unless specified otherwise; $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

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