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Stromal cells directly mediate the re-establishment of the lymph node compartments after transplantation by CXCR5 or CCL19/21 signalling

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Summary

Lymph nodes (LN) are highly organized and have characteristic compartments. Destruction of these compartments leads to an inability to fulfil their immunological function. However, it is not yet clearly understood which mechanisms are involved in the development and maintenance of this organization. After transplantation of LN into the mesentery, the LN regenerate to fully functional LN. In this study, the question was addressed, how stromal cells in the B-cell follicles (follicular dendritic cells), which were identified by CD21/CD35, and stromal cells in the T-cell area (gp38$^+$ cells) are involved via chemokine signalling. The gp38$^+$ cells and CD21/CD35$^+$ cells were detected in the transplanted LN (EGFP, plt/plt and CXCR5$^{-/-}$ mice) over a period of 8 weeks to analyse their competence to reconstruct the compartmental organization. The presence of gp38$^+$ cells was stable during regeneration and these cells reconstructed the T-cell area within 4 weeks. After transplantation of plt/plt LN CCL19/CCL21 expression was observed leading to partial restoration of the T-cell area. In contrast, there were changes in the presence and morphology of CD21/CD35$^+$ cells within the B-cell area during reconstruction, which was dependent on the presence of B cells and CXCL13/CXCR5 signalling. Hence, CD21/CD35$^+$ cells and gp38$^+$ cells are involved in the establishment of the compartmental organization of lymph nodes but using different ways to recruit lymphocytes via chemokine signalling.

Keywords: CCL19/CCL21; CXCR5; fibroblastic reticular cells; follicular dendritic cells; lymph node; regeneration

Introduction

Lymph nodes (LN) are located preferentially at sites where pathogenic antigens might invade the host. These antigens are transported as soluble antigens or by dendritic cells (DC) via the afferent lymphatics into the LN. Hence, the LN are directly connected to their draining area. This is an optimal position in which to act as a site where immune responses are induced.[1] To fulfil this function the structure is highly organized: naive T cells, which mainly arrive via the high endothelial venules, migrate into the T-cell area, the paracortex, where they meet DC and are stimulated by specific antigens. The B cells are mainly located in the follicular area of the cortex. Stimulation and affinity maturation of B cells is initiated in B-cell follicles. The third compartment is the medulla, where lymphocytes exit the LN via efferent lymphatics. Until recently, the dynamics of the immune cells were mainly studied. However, the non-haematopoietic cells, the stromal cells, which form the skeletal backbone of the LN[2,3] by forming a network and extracellular matrix components, may also be of functional relevance in immune reactions. It has become clear that these stromal cells have to be divided into subpopulations. Fibroblastic reticular cells (FRC) are one of various subtypes of stromal cells located in the T-cell area. These cells can be visualized by the antibody gp38, which identifies podoplanin. In addition, they are also CD31 negative.[4,5] Recently, it was shown that T cells migrate along the FRC fibres within the T-cell area,[6,7] documenting a potential relevance of such stromal cell–T-cell interactions.

It is well established that CCL19 and CCL21 are chemokines that are produced in secondary lymphoid organs. The T and B cells and also DC express the

chemokine receptor CCR7, which is the receptor for CCL19/CCL21. Signalling through this pathway leads to immigration of lymphocytes into the LN via high endothelial venules.[8] As it was shown that FRC produce CCL19/CCL21, it is likely that signalling through CCL19/CCL21 mediates the migration along the FRC.[5] However, whether these stromal-cell-produced chemokines facilitate the maintenance and reconstruction of the T-cell area has not been studied. In addition, it is not known whether FRC are the relevant cells which are able to recruit T cells into the T-cell area maintaining this compartment.

Follicular dendritic cells (FDC) are potent stimulators of B-cell maturation.[9] They are located in the B-cell area, especially in B-cell follicles. They are interconnected and form a dense network within the B-cell follicles. However, it has not been studied how FDC are involved in forming and maintaining B-cell follicles or the B-cell area. Several markers for FDC identification in different maturation stages exist. In the current study, CD21/CD35 expression and false-negative staining of B lymphocytes were used. From studies with $CXCR5^{-/-}$ mice it is known that the development of the lymphoid organs is dependent on signalling through the CXCL13/CXCR5 pathway.[10] During organogenesis so-called lymphocyte-inducer cells ($CD45^+$, $CD3^-$, LTa^+, Lti) interact with stromal organizer cells, which leads to up-regulation of adhesion molecules of these stromal-like cells. In addition, these organizers produce CXCL13 to recruit CXCR5-positive B cells forming the B-cell area of lymphoid organs.[11,12] However, it is unclear whether FDC assume this function of organizer cells after birth and how the B-cell area persists during adulthood.

Recently, a model was established to explore FRC and FDC in the *in vivo* context:

After removing the mesenteric lymph nodes (mLN) avascular donor LN were transplanted into the draining area of the intestine.[13] The LN tissues were reconnected to the afferent lymphatics and to the bloodstream and completely re-organized. The immune cells were substituted by host immune cells, coming mainly from peripheral sites. More importantly, the transplanted stromal cells persisted in the regenerated LN. Hence, only stromal cells from donor LN formed the skeletal structure of the regenerated LN in the draining area of the intestine. Therefore, this model can be applied to examine for the first time how stromal cells are involved in constructing and maintaining the compartmental structure of LN during life.[13]

The current study uses the described model by transplanting enhanced green fluorescent protein (EGFP) LN into wt mice on the one hand. By this, reconstruction of the LN was analysed over a period of 4 weeks. Moreover, the involvement of $gp38^+$ cells and $CD21/CD35^+$ cells was studied in detail. On the other hand, plt/plt mice [paucity of lymph node T cells (plt)][14] and $CXCR5^{-/-}$ mice[10] were used either as donors or recipients in this model to answer the question of whether signalling through the CXCR5/CXCL13 and CCL19/CCL21/CCR7 pathway is directly involved in maintaining the compartmentalization of the B-cell and T-cell areas.

Materials and methods

Animals

Female C57BL/6 and C57BL/6 plt/plt, B6.Cg-Blr1tm1 Lipp/J backcrossed for at least 15 generations to C57BL/6 mice (designated here as CXCR5-deficient mice; kindly provided by R. Foerster, Institute of Immunology, Hannover Medical School, Germany) and the transgenic C57BL/6-Tg(ACTbEGFP) (designated here as EGFP mice; these mice constitutively express EGFP in all cells under the control of the chicken β-actin promoter) were bred at the central animal laboratory of Hannover Medical School and were used at a weight of 18–25 g. All animal experiments were performed in accordance with the institutional guidelines and had been approved by the Niedersächsisches Landesamt für Verbraucherschutz und Lebensmittelsicherheit (No 33-42502-05/960).

Intestinal surgery

Mesenteric LN from EGFP mice were isolated and disrupted. Under combined anaesthesia with Ketamine (Gräub AG, Bern, Switzerland) and Domitor (Pfizer, Karlsruhe, Germany) the mLN of the small and large intestine of the host were excised and transplanted into C57BL/6 (wt) mice.[13] The recipients were killed 4, 7, 12 and 28 days after transplantation and the transplanted LN (LNtx) were analysed ($n = 3$ to $n = 5$, Table 1). Furthermore, mLN from plt/plt mice were used as donor LN and were transplanted into C57BL/6 mice (Table 1). In addition, mLN from wt mice were used as donor LN and were transplanted into $CXCR5^{-/-}$ mice (Table 1). After 8 weeks LNtx were removed and analysed ($n = 4$ to $n = 5$).

Flow cytometry for phenotyping stromal cells

Cell suspensions of pooled LN were made by treating them with 1 mg/ml collagenase 8 (Sigma, St Louis, MO) for 20 min at 37°. The enzyme activity was stopped by adding 10 mM EDTA to the cell suspension.

Cells were analysed in a FACS Canto (BD Biosciences, Heidelberg, Germany). About 1×10^6 cells were incubated with the monoclonal antibody CD45 phycoerythrin (PE) -Cy7 (BD Biosciences) to identify the non-haematopoietic stromal cells. These CD45 cells were stained by using Podoplanin-PE antibody (gp38; Biolegend, San Diego, CA) together with anti-CD31 (Biolegend, Uithoom, the Netherlands) and FDC was detected by CD21/CD35-allophycocyanin (BD Biosciences) staining. High endothelial venules were identified by the monoclonal

Table 1. Experimental design

Donor LN	Host	Time after transplantation	Nomenclature	Analysis of LN fragment
EGFP	C57BL/6	4, 7, 12 and 28 days	EGFP-tx	Survival of FRC and FDC presence and number of FRC, FDC and immune cells proliferation (Ki67)
plt/plt	C57BL/6	8 weeks	plt-tx	CCL19/21 expression compartment structure presence of FRC, FDC, immune cells and CCL21
C57BL/6	CXCR5$^{-/-}$	8 weeks	CXCR5-tx	Compartment structure presence of FRC, FDC and immune cells

EGFP, enhanced green fluorescent protein; FDC, follicular dendritic cells; FRC, fibroblastic reticular cells; LN, lymph node; plt, paucity of lymph node T cells.

antibody MECA79 and lymphatic endothelial cells by the LYVE-1 monoclonal antibody (abcam, Cambridge, MA). Donor stromal cells which survived the transplantation were identified by their EGFP signalling.

Flow cytometry for phenotyping immune cells

Cell suspensions from LNtx were made and about 1×10^6 cells were incubated with antibodies against T cells (CD3 biotinylated and revealed by streptavidin with peridinin chlorophyll protein; BD Biosciences) and B cells (CD19 coupled with allophycocyanin-H7; BD Biosciences). Double positive cells were not measured in this context.

Quantification of mRNA expression among mLNtx and pLNtx

Total RNA of LNtx and control LN ($n = 3$) was isolated according to the manufacturer's protocol (Rneasy kit; Qiagen, Hilden, Germany) and cDNA synthesis was performed with 50 mM oligo-primer, 0·1 M dithiothreitol, 5× first-strand buffer, 10 mM dNTP, 35 U/μl RNAse inhibitor and 200 U/μl Moloney murine leukaemia virus reverse transcriptase (all obtained from Invitrogen, Karlsruhe, Germany) in a total volume of 20 μl at 37° for 50 min. With this cDNA, quantitative real-time PCR was performed using the QuantiTect SYBR-Green protocol from Qiagen. The primer sequences and amplicon sizes were as follows: CCL19atg (5′-CTGCCTCAGATTATCTGCCAT-3′ and 5′-AGGTAGCGGAAGGCTTTCAC-3′) CCL21ser (5′-ATGTGCAAACCCTGAGGAAG-3′ and 5′-TCCTCTTGAG GGCTGTGTCT-3′; 178 bp[4]) and β-actin (5′-AGCCATGT ACGTAGCCATCC-3′ and 5′-CTCTCAGCTGTGGTGGT-GAA-3′; 228 bp; housekeeping gene).

Immunohistochemistry

Cryostat sections of control LN and LNtx (7 μm, $n = 4$ to $n = 5$) were fixed in acetone/methanol solution (1 : 1, 10 min, −20°). Immunofluorescence histochemistry was performed according to standard protocols.[15] Briefly, sections were rehydrated in TBST (0·1 M Tris–HCl pH 7·5, 0·15 M NaCl, 0·1% Tween-20) and pre-incubated with TBST containing 5% rat or mouse serum. The sections were stained with fluorescent dye-coupled antibodies gp38-Cy5 (kindly provided by R. Foerster, Institute of Immunology, Hannover Medical School, Germany), CD11c-FITC (DC; BD Biosciences) and antibodies against CCL21 (Serotec, Oxford, UK), CD21/CD35-allophycocyanin (FDC; BD Biosciences), B220-biotinylated (B cells, kindly provided by R. Foerster, Institute of Immunology, Hannover Medical School, Germany), anti-CD3-biotinylated (BD Biosciences) in 2·5% serum/TBST, which was visualized by a streptavidin coupled with Cy3 (Invitrogen). CCL21 was shown by using a goat anti-rabbit antibody coupled with Cy3 (Dianova, Hamburg, Germany). Nuclei were visualized by DAPI staining (1 μg/ml DAPI/TBST), and sections were mounted with Fluorescent Mounting Medium (Dako, Hamburg, Germany). Images were acquired using an Axiovert 200 M microscope with AXIOVISION software (Carl Zeiss, Jena, Germany). Proliferation of gp38$^+$ cells and CD21/CD35$^+$ cells was visualized by using the antibody against the Ki67 antigen (Epitomice, Burlingame, CA), which was detected by a goat anti-rabbit antibody coupled with Cy3 (Dianova). In addition, cell suspensions of these LN were made as described above. These cells were incubated with CD45 microbeads (Milenyi Biotec Ltd., Bergisch Gladbach, Germany). Then, the cells were isolated by the MACS technique (Miltenyi Biotec Ltd.) according to the supplied protocol. Afterwards, cytospins were carried out[16] and cells were stained as described above.

Data analysis

Calculations, statistical analysis and graphs were performed with the software GRAPHPAD PRISM 4.0 (Graphpad Software Inc., San Diego, CA). Statistical differences were calculated, performed in the unpaired *t*-test and are indicated by $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

Results

gp38$^+$ cells form the main pool of stromal cells and survive during regeneration

Non-haematopoietic cells were analysed by FACS analysis. As peripheral LN and mLN showed no differences in the

percentages in the subset composition (data not shown), pooled LN were used for the following analysis. Most of the CD45$^-$ cells were positive for podoplanin (gp38$^+$, 40 ± 3·8%, n = 3) and therefore visualized by this antibody (Fig. 1a). Some of the gp38$^+$ cells were also positive for CD31, showing that these cells have lymphatic properties (gp38$^+$ CD31$^+$; 6·8 ± 0·2%, n = 3). For the following analysis gp38$^+$ cells were used. The FDC were identified and visualized by detection of CD21/CD35 expression among the CD45$^-$ population. To strengthen the CD21/CD35 marker to identify FDC, B cells were excluded by false-negative staining of B cells and staining against CD157 cells (BST-1 or BP-3[17]; data not shown). CD21/CD35$^+$ cells represented up to a quarter of stromal cells (20 ± 5·5%, n = 5), being the second most frequent subpopulation of stromal cells (Fig. 1a). The remaining population of CD45$^-$ cells included endothelial cells of blood vessels (e.g. MECA79$^+$ cells, 11·1 ± 0·3%, n = 3) and lymphatic endothelial cells (LYVE-1$^+$ cells, 22·6 ± 0·9%, n = 3).

It was examined, whether gp38$^+$ cells and CD21/CD35$^+$ cells were able to survive during regeneration and were responsible for re-establishment of the compartments of the transplanted LN. Therefore, after removing the mLN of wt mice, EGFP$^+$ LN were implanted into this area (EGFP-tx). Hence, by detecting the EGFP signal among the gp38$^+$ and CD21/CD35$^+$ cells, it was documented whether these cells were of donor or host origin. Most of the gp38$^+$ cells being in the transplanted LN were detected as EGFP$^+$ cells after regeneration compared with the control (Fig. 1b). The data suggest that gp38$^+$ cells survived during regeneration or regenerated from the EGFP$^+$ gp38$^+$ cells (Fig. 1b). Interestingly, there are two populations of gp38$^+$ cells which are low and high positive for EGFP$^+$ (Fig. 2a).

Focusing on the CD21/CD35$^+$ cell population in the B-cell follicles after regeneration, Fig. 1 shows that both EGFP$^+$ and EGFP$^-$ CD21/CD35$^+$ cells were present. Hence, fractions of donor CD21/CD35$^+$ cells were lost within the transplanted LN, and host FDC repopulated the LN.

Next, to what extent EGFP$^+$ gp38$^+$ cells and CD21/CD35$^+$ cells were present for a period of 28 days during regeneration was analysed by flow cytometry Figure 2(a) shows that, as was shown histologically, most gp38$^+$ cells in the transplanted LN were detected as EGFP$^+$ cells after regeneration. In addition, most gp38$^+$ cells were EGFP$^+$ at all observed time-points.

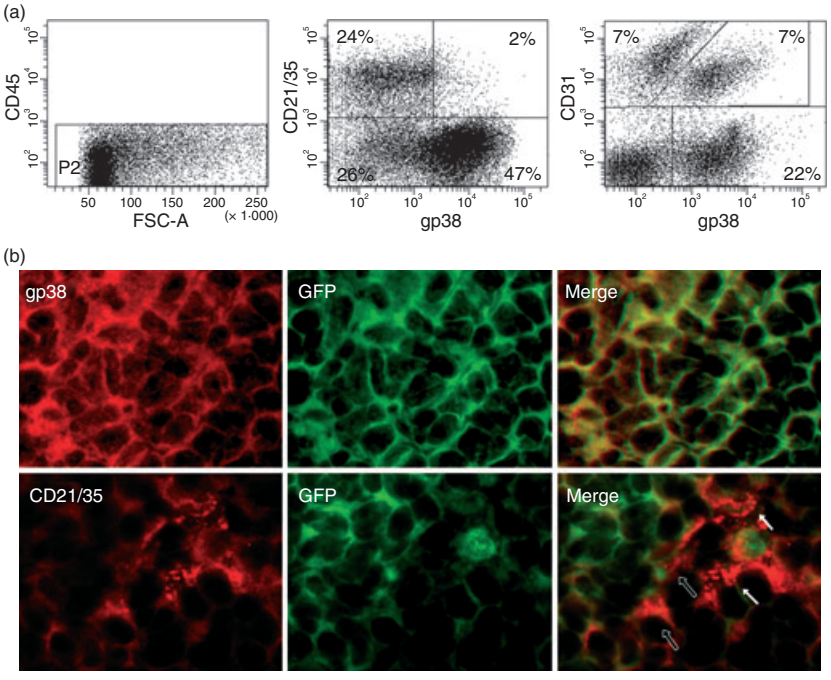

Figure 1. Differential regeneration of stromal cell subsets in lymph nodes (LN). (a) LN of wild-type (wt) mice were removed and cell suspensions were stained and analysed by flow cytometry. Only viable cells were gated and the dot plots show CD45$^-$ cells [y-axis, x-axis forward scatter (FSC)], which were used to detect gp38$^+$ and CD21/CD35$^+$ cells, which are shown in the second dot plot. On the right hand side a dot plot is shown, where CD45$^-$ cells were stained with gp38$^+$ and CD31, which is a vascular marker. The percentages are representative for three to five independent experiments. (b) gp38$^+$ and CD21/CD35$^+$ cells are seen in red and the remaining donor EGFP$^+$ cells in the transplanted LN (EGFP-tx) in green. The merged figure shows that CD21/CD35$^+$ cells are both EGFP$^+$ (open arrows) and EGFP$^-$ (closed arrows) illustrating that these cells are partly donor-derived after regeneration (8 weeks after transplantation). In contrast, most gp38$^+$ cells are EGFP$^+$ 8 weeks after transplantation.

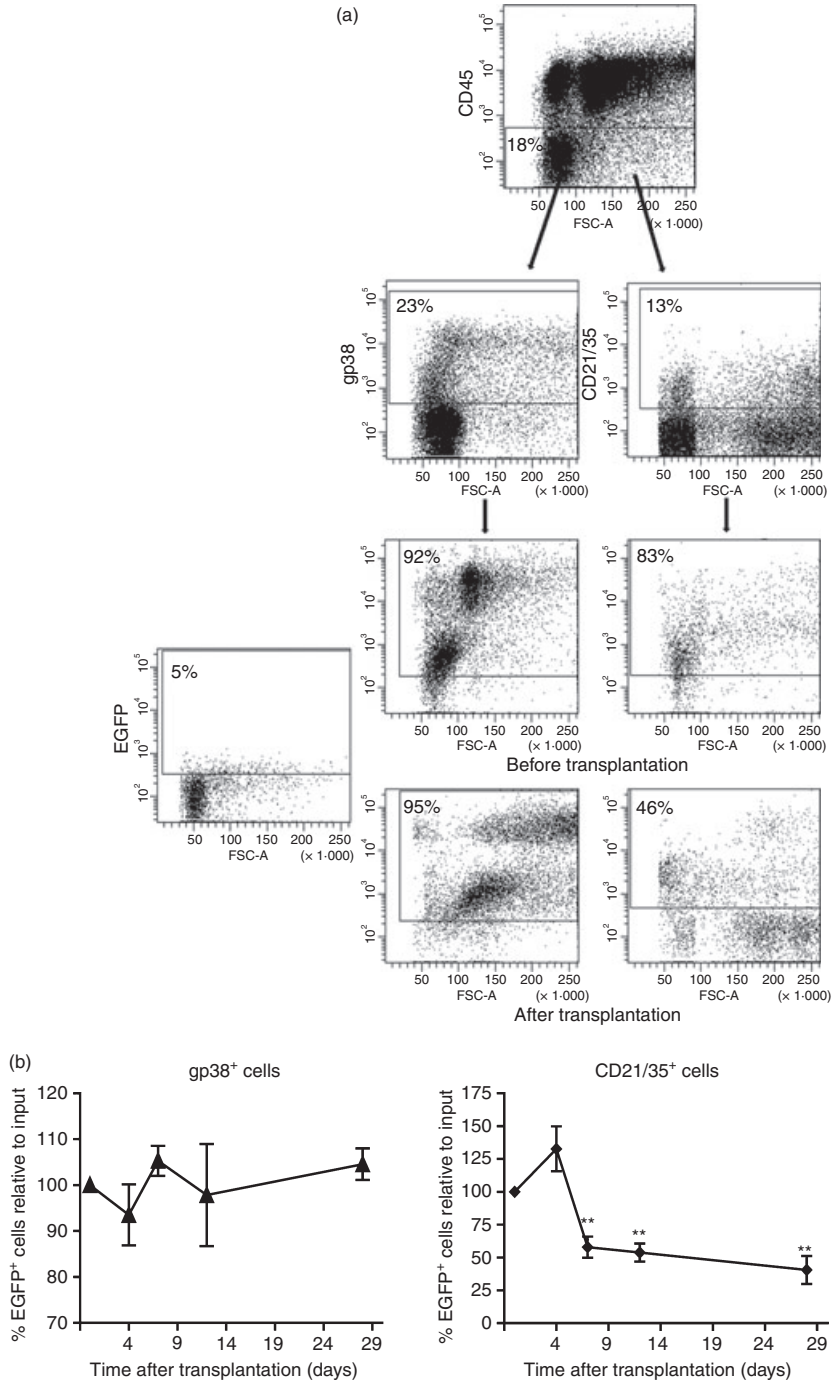

Figure 2. The presence of endogenous gp38$^+$ cells is stable during reconstruction of the lymph nodes (LN). (a) Enhanced green fluorescent protein (EGFP) mesenteric LN (mLN) were transplanted into the mesentery of wild-type (wt) animals after removal of the endogenous mLN (EGFP$^-$tx). The LNtx were removed 28 days after transplantation and analysed by flow cytometry. The dot plots show the CD45 staining. By gating on the CD45$^-$ population the gp38$^+$ and CD21/CD35$^+$ cells were detected. Then, the EGFP signal was analysed on CD45$^-$ gp38$^+$ cells and on CD45$^-$ CD21/CD35$^+$ cells before transplantation and 28 days after transplantation. The dot plots are representative of three experiments. The EGFP signal among CD45$^-$ gp38$^+$ and CD45$^-$ CD21/CD35$^+$ cells of EGFP-tx was detected in C57/BL6 mice and served as a control. Then, the EGFP signal was measured before and 28 days after regeneration. (b) The number of EGFP$^+$ cells 4, 7, 12 and 28 days after transplantation was calculated as the percentage of the initial EGFP cell population. The graph shows means and standard error of the relative percentage of EGFP$^+$ cells after transplantation ($n = 3$) and significant differences in the unpaired t-test are indicated by $^{**}P < 0{\cdot}01$. While the percentage of EGFP$^+$ cells remained stable among the gp38$^+$ cell population, EGFP$^+$ cells among the CD21/CD35$^+$ cell population were diminished during regeneration.

In contrast, CD21/CD35$^+$ cells showed different kinetics: 4 days after transplantation the EGFP signal had declined to only 50% among the detected CD21/CD35$^+$ cells, which was stable over the later time-points (Fig. 2a,b).

gp38$^+$ cells show stable morphology and presence during regeneration of the LN

Next, it was clarified whether the remaining gp38$^+$ cells were sessile during regeneration or whether there was a new formation of the gp38$^+$ cell network. Therefore, where gp38$^+$ cells were located within the tissue during regeneration was analysed. At early time-points (4 and 7 days) islets of T-cell areas were seen, whereas later and after regeneration (12 and 28 days) a large T-cell area was observed (Fig. 3a). Focusing on the distribution of gp38$^+$ cells, it was also seen that small densely clustered gp38$^+$ cell networks were located through the tissue early after transplantation (Fig. 3a). A large developed gp38$^+$ cell network, which was consistent with the extension of the T-cell area, then appeared later during regeneration (Fig. 3a). Hence, the existing gp38$^+$ cells formed a new network which led to reconstruction of the T-cell area.

As we showed that the immune cells of the transplanted LN were mainly repopulated by host immune cells, whether these cells were able to influence the presence of gp38$^+$ cells in the transplanted LN. At early time-points after transplantation the presence of CD3$^+$ T cells was dramatically reduced compared with a normal LN. However, at later time-points the percentage of CD3$^+$ T cells returned to normal levels (Fig. 3b) and was EGFP$^-$. This documents that host T cells repopulated the transplanted LN. Furthermore, the maintenance of gp38$^+$ stromal cells was analysed 4, 7, 12 and 28 days after transplantation. The data showed an increase in percentage of gp38$^+$ cells 7 days after transplantation (Fig. 3b). However, the percentage of gp38$^+$ cells did not change concerning the beginning and the end of the observed time-points. Hence, the data suggest that the influx of CD3$^+$ cells had no effect on the presence of gp38$^+$ cells.

CD21/CD35$^+$ cells are absent in the transplanted LN at early time-points after transplantation

Next, we focused on the presence and localization of CD21/CD35$^+$ cells within the transplanted LN during the process of regeneration. Surprisingly, during early time-points after transplantation CD21/CD35$^+$ cells were hardly detectable in the immunohistology (Fig. 4a). In addition, the FDC identification by the marker FDC-M1 also failed (data not shown). However, the presence of CD21/CD35$^+$ cells was shown to remain stable over the observed time-points using flow cytometry (Fig. 4b).

B cells were distributed over the whole transplanted tissue, and B-cell area and secondary follicle formation were not seen at early time-points. However, later and after regeneration CD21/CD35$^+$ cells were relocated in B-cell follicles forming and reconstructing the B-cell areas of the regenerated LN (Fig. 4a). As it was shown in Fig. 2 that CD21/CD35$^+$ cells were also EGFP$^-$, the data indicate that host-derived CD21/CD35$^+$ cells reconstructed the B-cell area of the regenerated LN in contrast to the gp38$^+$ cell population.

Similar to the CD3$^+$ T cells, the percentage of B cells in the transplanted LN was diminished at early time-points after transplantation, but host-derived B cells repopulated the regenerated LN at later time-points (Fig. 4b). Hence, reconstruction of the B-cell compartment occurred in parallel with the reformation of the CD21/CD35$^+$ cell network and the presence of B cells.

gp38$^+$ cells and CD21/CD35$^+$ cells are able to proliferate during reconstruction of the LN

As the data showed that gp38$^+$ cells increased 7 days after transplantation and the EGFP signal did not change during regeneration, it is likely that gp38$^+$ cells are able to proliferate. Hence, the proliferative capacity of gp38$^+$ cells and also of CD21/CD35$^+$ cells was analysed by identifying the Ki67 antigen using fluorescence microscopy both in the intact tissue and on cytospins, where CD45$^-$ cells were isolated. The gp38$^+$ cells and the CD21/CD35$^+$ cells did not express the Ki67 antigen in normal LN (Fig. 5), documenting that these stromal cells were not in the cell cycle under steady-state conditions. Interestingly, during reconstruction of the LNtx compartments the Ki67 antigen was detected among the gp38$^+$ cells and CD21/CD35$^+$ cells (Fig. 5). Hence, the presence in the cell cycle was important in the homeostasis of the presence of stromal cells.

The presence of peripherally derived CCL19/CCL21 is responsible for the formation of the T-cell compartments by influencing gp38$^+$ cells

Next, it was investigated whether these chemokines CCL19/CCL21 were able to modulate the competence of gp38$^+$ cells to reconstruct the T-cell compartments during regeneration. Therefore, LN of plt/plt mice were transplanted into wt mice (plt-tx). After regeneration the expression of CCL19/CCL21 was observed in the whole regenerated LN (plt-tx) compared with wt and plt LN using real-time PCR.

As expected, LN of plt/plt mice failed to produce CCL19/CCL21 mRNA (Fig. 6a), whereas LN of wt mice produced high levels of CCL19/CCL21 (Fig. 6a). Interestingly, transplanted plt-tx LN showed an intermediate level of CCL19 expression and a lower level of CCL21

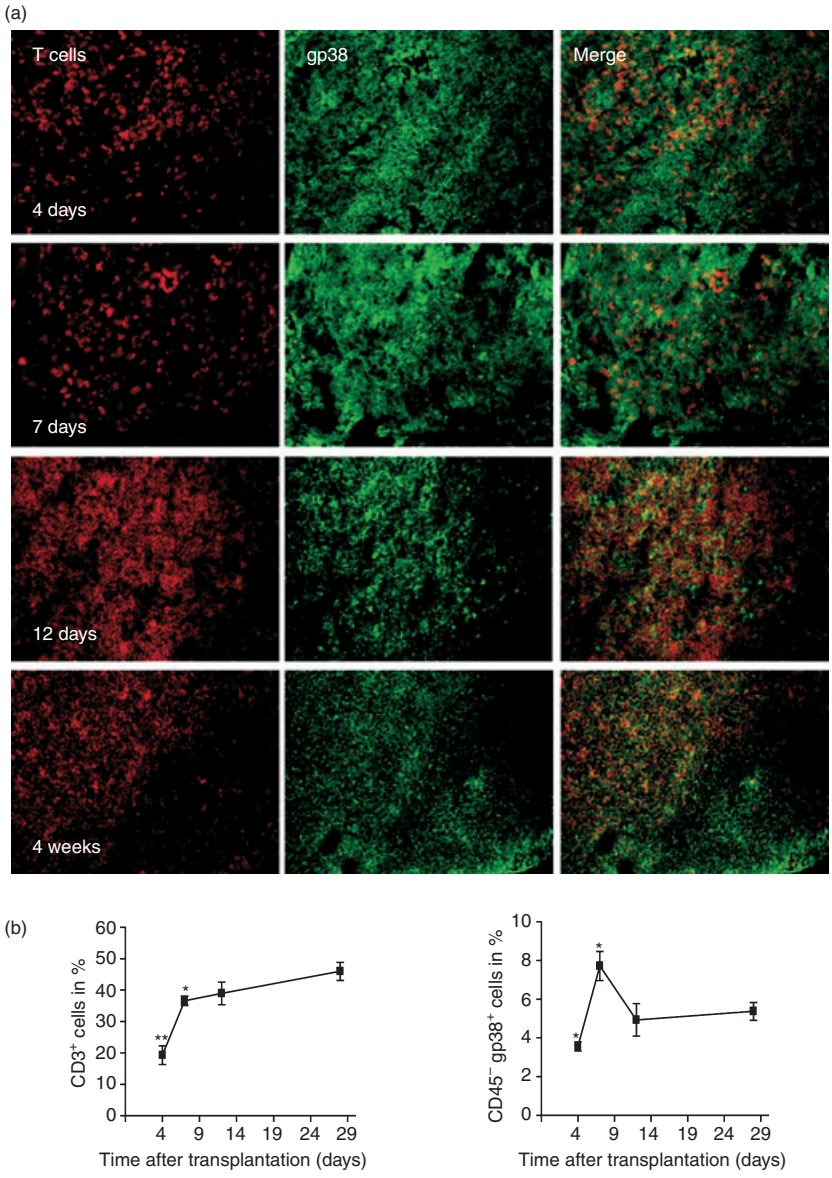

Figure 3. gp38$^+$ cells are able to re-organize their network. (a) Re-organization of the compartments after transplantation was analysed by detection of T cells via the anti-CD3 antibody and detection of gp38$^+$ cells by fluorescence microscopy. T cells are shown in red and gp38$^+$ cells in green. At early time-points after transplantation (4 and 7 days) the T-cell compartment is destroyed. T cells are distributed over the whole lymph node (LN), whereas at later time-points (12 and 28 days) T cells are again clustered in the T-cell compartment. gp38$^+$ cells are clustered at early time-points, whereas at later time-points the network of the T-cell compartment is again fully developed. Hence, gp38$^+$ cells form a new network which leads to re-construction of the T-cell area. (b) The percentage of CD3$^+$ T cells and CD45$^-$ gp38$^+$ cells among the transplanted LN (LNtx) cells was analysed via flow cytometry during regeneration. Means and standard error are given ($n = 3$) and significant differences in the unpaired *t*-test are indicated by $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$. The data show that the T-cell population is decreased at early time-points after transplantation, but is restored after reconstruction. In contrast, the percentage of the CD45$^-$ gp38$^+$ cell population remains the same after regeneration with a peak on day 7. Hence, the influx of CD3$^+$ cells has no effect on the presence of gp38$^+$ cells.

expression (Fig. 6a), indicating that CCL19/CCL21 were re-expressed in the regenerated plt-tx LN.

Next, the formation of the compartments was analysed in wt, plt/plt and plt-tx LN. Compared with the wt LN, the LN of plt/plt mice exhibited a totally destroyed T-cell compartment. T cells were loosely distributed over the whole LN (Fig. 6b). However, the B-cell area formation was unaffected by the absence of CCL19/CCL21. In line with the CCL19/CCL21 re-expression after regeneration of the plt-tx LN, the T-cell compartment of this LN was reconstructed (Fig. 6b). Obviously, the formation of the T-cell area was dependent on CCL19/CCL21 expression and was connected to the gp38$^+$ cell network formation.

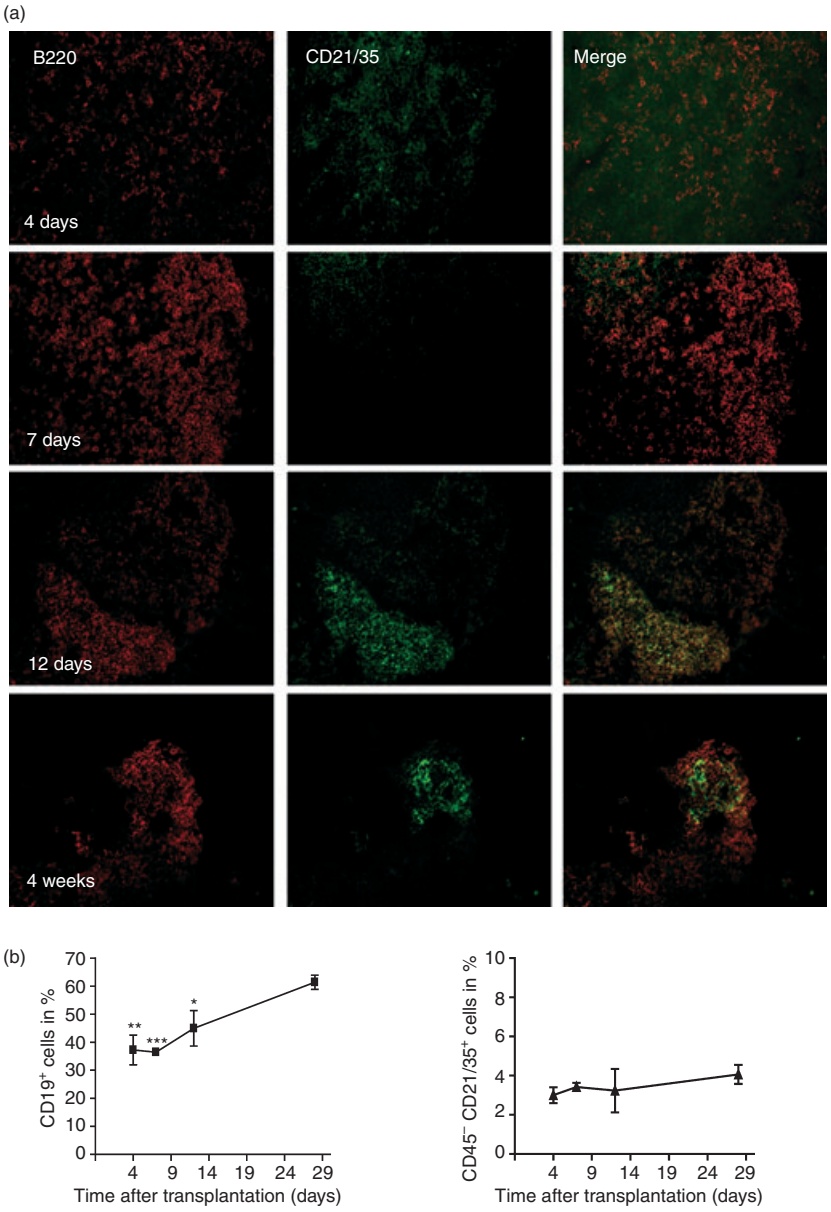

Figure 4. CD21/CD35$^+$ cells are hardly detectable at early time-points after transplantation. (a) Re-organization of the compartments after transplantation was analysed by detection of B cells via the anti-B220 antibody and detection of CD21/CD35$^+$ cells by fluorescence microscopy. B cells are shown in red, CD21/CD35$^+$ cells in green. At early time-points after transplantation (4 and 7 days) the B-cell compartment is destroyed and B-cell follicles are no longer seen. At late time-points B cells (12 and 28 days) are again clustered in the B-cell compartment and B-cell follicles are seen. In contrast to gp38$^+$ cells, CD21/CD35$^+$ cells are hardly detectable at early time-points by fluorescence microscopy. Some CD21/CD35$^+$ cells are distributed over the whole transplanted LNtx on days 4 and 7. However, at later time-points CD21/CD35$^+$ cells are again seen in the B-cell compartment forming a new network and B-cell follicles. (b) The percentages of B cells were analysed using the CD19 monoclonal antibodies coupled with allophycocyanin-H7 by flow cytometry during regeneration among the transplanted lymph node (LNtx) cells and follicular dendritic cells (FDC) via gating on the negative population of CD45 cells, which expressed CD21/CD35. Means and standard error are given ($n = 3$) and significant differences in the unpaired t-test are indicated by $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$. The data show that the B-cell population is decreased at early time-points after transplantation, but is restored after reconstruction. In contrast to analysis in fluorescence microscopy, the CD21/CD35$^+$ population is detectable at early time-points after transplantation by flow cytometry and remains stable during regeneration.

Interestingly, detection of the CCL21 protein revealed that CCL21 was localized near the gp38$^+$ cells as well as DC in the wt control (Fig. 7). This co-localization was shown several times in the T-cell area in these mice (Fig. S1). As expected, CCL21 was not seen in plt/plt mice. In line with the real-time PCR data, the protein CCL21 was detectable after regeneration in plt-tx and localized with both the gp38$^+$ cells and DC (Fig. 7).

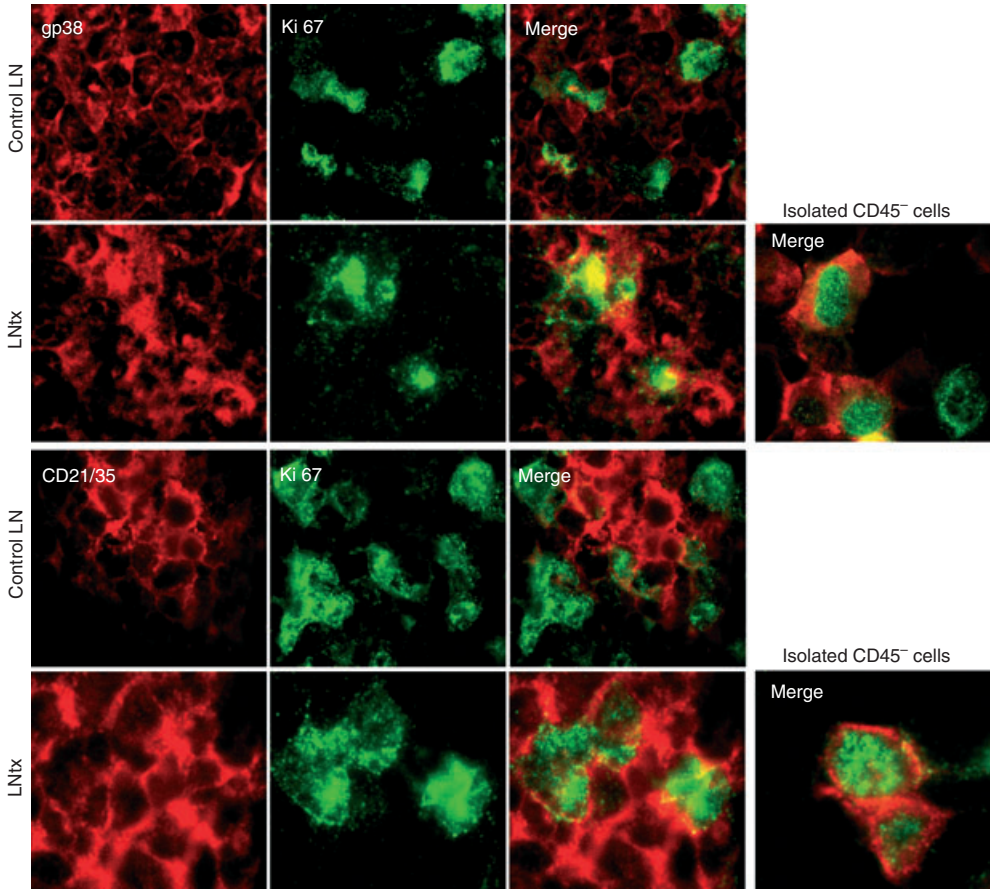

Figure 5. gp38$^+$ and CD21/CD35$^+$ cells proliferate during reconstruction of the compartments. Proliferation of CD21/CD35$^+$ cells and gp38$^+$ cells (red) was detected by the Ki67 antigen (green) in an untreated lymph node (LN), and after transplantation on day 7, both in an intact transplanted LN (LNtx; cryostat section) and CD45$^-$ cells isolated from LNtx (cytospin). The merged illustrations show that both CD21/CD35$^+$ and gp38$^+$ cells proliferate during regeneration and not under steady-state conditions. This proliferative capacity is observed during the whole regeneration. However, proliferation peaks on day 7, which is shown here.

CXCR5/CXCL13 signalling is directly involved in the formation and function of the B-cell area

To elucidate, which functional and molecular mechanism might be behind the reformation of the CD21/CD35$^+$ cell network and the presence of B cells, wt LN were transplanted into CXCR5$^{-/-}$ mice (CXCR5-tx). Here, CD21/CD35$^+$ cells of wt LN were able to synthesize the chemokine CXCL13 to attract B cells. However, B cells were not able to respond to these signals because they lacked the receptor CXCR5. It was therefore questioned whether this scenario had any influence on the regeneration of the transplanted LN. The LN of CXCR5$^{-/-}$ mice showed impaired B-cell compartments. B cells were distributed over the whole LN and defined B-cell follicles were not seen in these mice (Fig. 8). Interestingly, after transplantation of wt LN into CXCR5$^{-/-}$ mice the regenerated LN (CXCR5-tx) exhibited the same phenotype as described for CXCR5$^{-/-}$ LN: B-cell follicles were not seen in these CXCR5-tx after 8 weeks (Fig. 8b). In addition, the CD21/CD35$^+$ cell network of CXCR5$^{-/-}$ and CXCR5-tx was also destroyed and not comparable to wt LN (Fig. 8). The data showed that CXCL13/CXCR5 signalling was critical for the reconstruction of the LN.

Discussion

It has long been known that LN are highly organized organs, where immune cells are able to effectively interact with each other, the T-cell area being packed with T cells and DC. Most of these cells are highly mobile, migrating within the tissue to find their specific antigen.[18] Since 2008 it became clear that in addition to these highly motile cells, subpopulations of stromal cells are positive for podoplanin (gp38) namely FRC and marginal reticular cells.[19] Whereas FRC are located in the T-cell area and marginal reticular cells are near to the marginal sinus, both are CD31 negative.[4,19] The FRC are important components, forming the structure of the LN by producing extracellular matrix components.[7,17] T cells are able to migrate along the FRC network and further immunological functions, e.g. homeostasis of the T

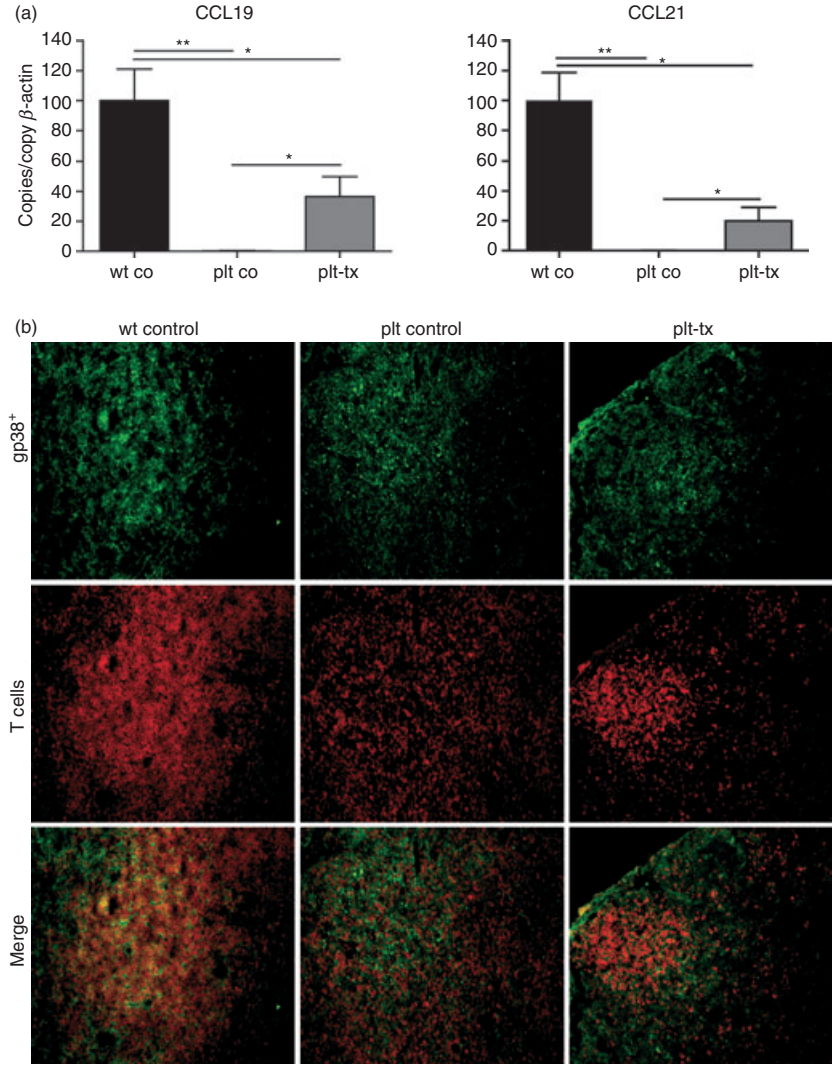

Figure 6. Reconstruction of the T-cell area is dependent on CCL19/CCL21 expression. (a) Wild-type (wt), plt/plt and plt-tx lymph nodes (LN) were excised 8 weeks after transplantation, the mRNA was isolated and real-time PCR was performed in triplets for CCL19atg and CCL21ser. The data are normalized to the housekeeping gene β-actin (copies/copy β-actin). Means and standard error are given ($n = 4$ to $n = 8$). Significant differences in the unpaired t-test are indicated by $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}001$. Regenerated plt-tx LN show re-expression of CCL19atg and CCL21ser. (b) The structure of the T-cell compartment of wt, plt/plt and plt-tx LN was analysed. plt-tx LN show a comparable distribution and network building to wt LN after their reconstruction (8 weeks after transplantation). gp38^{+} cells are seen in green, T cells in red (anti-CD3 monoclonal antibodies). The T-cell compartment of plt LN is destroyed. Plt-tx LN that were transplanted into wt animals show a reconstruction of the T-cell area.

cell pool, have been described.[6] In contrast to the T-cell area, a part of the extracellular matrix component structures (e.g. conduits) of the B-cell area is primarily formed by FRC and then engaged by FDC.[20] FDC in the B-cell area and predominantly in the germinal centres form an interconnected network. Recently, it was underlined that FDC are key players in the induction of B-cell responses.[17] Similar to T cells, B cells also constitutively migrate into the B-cell area to increase the chance of encountering antigen.[9]

The current study deals with the question whether these stromal cell populations, FRC and FDC, are actively involved in the re-establishment and maintenance of the compartments and whether they act in the same way. Are FRC and FDC able to reconstruct a T-cell and B-cell area during regeneration in an adult, and which mechanisms regulate this? To answer this question, the recently established *in vivo* model was used, in which a LN was transplanted into the mesentery (LNtx) after removal of the original mLN.[13] Previous studies showed that early after transplantation the compartment structures of LNtx are destroyed. However, the LNtx regenerate with normal compartment structures, afferent lymphatics, blood supply and functional competence like control LN.[13] The

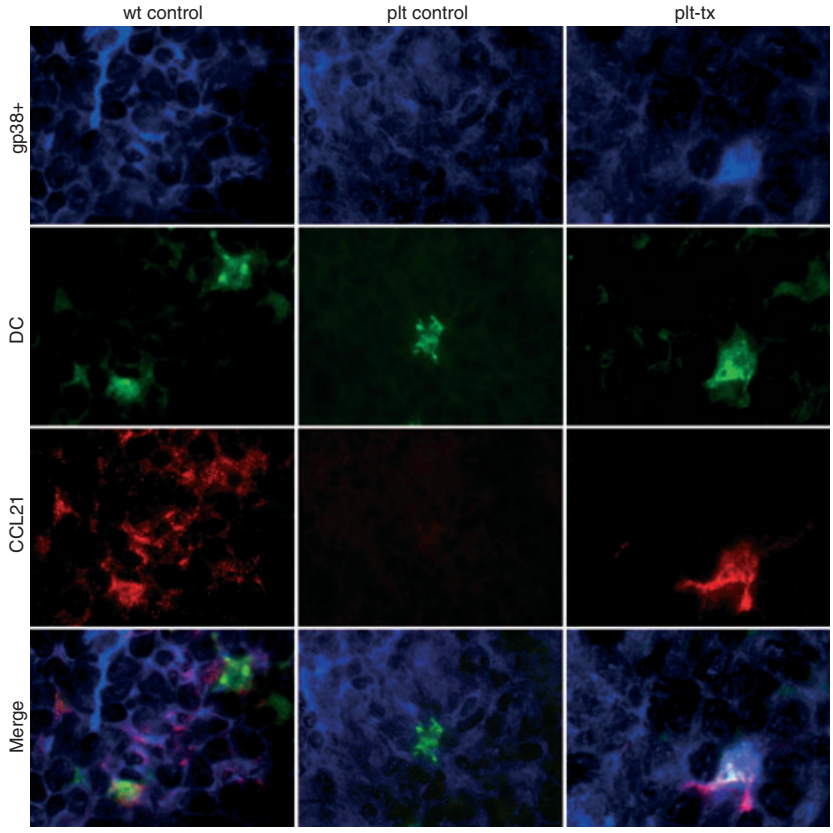

Figure 7. Dendritic cells (DC) are responsible for the re-expression of CCL21 in plt-tx. In a wild-type (wt) control CCL21 (red) is located near to the gp38$^+$ cells (blue) and DC (green). In the plt control CCL21 is completely absent. Interestingly, the merged pictures show that re-location of CCL21 near to DC and gp38$^+$ cells (pink) was seen after transplantation and regeneration of plt-tx.

current study showed that the percentage of gp38$^+$ cells was not affected by the process of regeneration. In line with this observation the data showed that most of the gp38$^+$ cells were of donor origin (EGFP$^+$). This is in contrast to Hammerschmidt *et al.*,[21] in which only 50% of the gp38$^+$ cells were found to be of donor origin. However, it was shown in detail by immunohistology that nearly all gp38$^+$ cells were from the donor,[13] which is in line with the current observation. The differing results may derive from different breeding procedures or from differences in analysing these cells. gp38$^+$ cells or their precursors did not migrate from the periphery into the transplanted LN, but remained in the LNtx. Endogenous proliferation within the LNtx seems to be responsible for the maintenance of the stable presence of gp38$^+$ cells, which may lead to the different EGFP expression capacity of these cells after transplantation. This is in line with data after lymphocytic choriomeningitis virus (LCMV) infection of mice: after destruction of the compartment by LCMV infection FRC are able to reconstruct the T-cell area of the spleen.[22]

Clusters of the gp38$^+$ cell network were present during the whole period of regeneration. However, only at later time-points after transplantation was a fully developed T-cell area seen including the gp38$^+$ cell network and T cells. This clearly shows that FRC are able to re-organize themselves to form an interconnected network, and to interact within the LN to interconnect.

The current study shows that the percentage of the CD21/CD35$^+$ FDC within the tissue is also stable during reconstruction of the LN. However, in contrast to the gp38$^+$ cells, host-derived FDC repopulate the regenerated LN, as shown by the EGFP cells. Hence, precursors of FDC from the periphery are directly involved in the reconstruction of the B-cell area and B-cell follicles. Interestingly, during early time-points after transplantation, CD21/CD35$^+$ cells were hardly detectable by immunofluorescence histology. This suggests that either LN-transplanted CD21/CD35$^+$ cells or immigrant precursors of CD21/CD35$^+$ cells partially lose their CD21/CD35 expression, which is hardly detectable in the histology but can still be measured in the flow cytometry. In addition, the dispersed localization of these cells in the LN early after transplantation may limit the detection in the histology. However, CD21/CD35$^+$ cells were re-established after reconstruction. Hence, the recruitment and migration of FDC to re-establish the B-cell compartment is not restricted to the LN itself, but rather extended to the periphery. However, our data showed for the first time

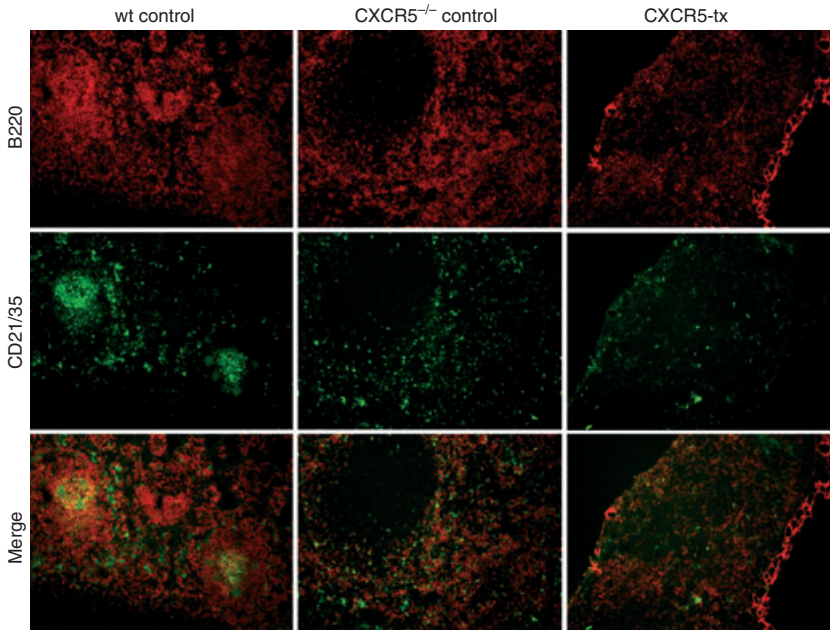

Figure 8. CXCR5/CXCL13 signalling is important for the reconstruction of the B-cell compartment. Wild-type (wt) lymph nodes (LN) were transplanted into $CXCR5^{-/-}$ mice (CXCR5-tx) to examine the role of CXCR5/CXCL13 signalling during reconstruction of the B-cell compartment (8 weeks after transplantation). Compared with the wt control, the distribution of the $B220^{+}$ cells (B cells, red) is impaired in $CXCR5^{-/-}$ LN. In addition, the presence and the network of $CD21/CD35^{+}$ cells (green) are also destroyed and not comparable to wt LN. The merge illustration shows that B cells and $CD21/CD35^{+}$ cells are not organized by each other. In line with these data, wt LN transplanted into $CXCR5^{-/-}$ mice show a comparable disorganization of the B-cell compartment.

that FDC are also able to proliferate within the tissue so the presence of FDC may also be achieved by replication. This occurs during the process of regeneration, which was not recorded until now in a normal LN.

CCL19 and CCL21 are important chemokines to attract T and B cells into the LN. Immune cells bearing the CCR7 receptor are able to migrate into the secondary lymphoid organs.[8] It has been shown that not only stromal cells but also lymphatic endothelial cells and high endothelial venules are producers of these cytokines, having a central role in guiding immune cells into a distinct compartment.[5,23] As the current and recently published data showed that FRC and high endothelial venules remained donor-derived, these cells are not able to express CCL19/CCL21 in plt/plt mice. Interestingly, re-expression of CCL19/CCL21 was found after transplantation and regeneration of plt/plt LN in wt mice (plt-tx). Furthermore, the disorganized T-cell area of the plt/plt LN was reconstructed after regeneration. Hence, this strongly suggests that the re-established CCL19/CCL21 expression in the regenerated plt-tx is a key player in mediating the reconstruction of the T-cell area. However, FRC do not seem to be the only source of CCL19/CCL21 expression in this situation. It was shown that DC are able to produce small amounts of CCL19.[5] Hence, as most DC were host-derived, coming from the draining area into the regenerated LN, these cells are candidates for the re-expression of CCL19/CCL21 within the regenerated LN. In addition, it has been shown that stromal cells are able to bind chemokines via the CCX-CKR1 (silent chemokine receptor), which can bind these chemokines without inside signalling events.[24] In addition, other extracellular matrix components are able to bind chemokines.[25] However, the regenerated LNtx are re-connected to the afferent lymphatics. For this reason host-derived lymphatic endothelial cells have to grow into the transplanted tissue. Hence, in addition to the co-localization of DC with CCL21, the effect of the regenerated plt-tx can also be mediated by the lymphatic endothelial cells. Our data show that CCL21 was present in the vicinity of DC and plt-tx $gp38^{+}$ cells, which were located close to the DC. This suggested that CCL21 was produced by DC and then fished by $gp38^{+}$ cells, which may lead to a reconstruction of the T-cell compartment.

B cells continuously migrate from the periphery into LN. They were recruited from the periphery after transplantation to migrate into the B-cell area or to become activated in the secondary follicle.[13] In the present study, both B cells and the $CD21/CD35^{+}$ cells of the regenerated wt LN, transplanted into $CXCR5^{-/-}$ mice (CXCR5-tx), were disorganized and distributed over the whole LN. B-cell areas and B-cell follicles were not formed. As wt LN were transplanted into $CXCR5^{-/-}$ mice, $CD21/CD35^{+}$ cells are not able to form a network and attract host-derived $CXCR5^{-/-}$ B cells. The forming of the $CD21/CD35^{+}$ cell network to re-construct the B-cell area and B-cell follicles is closely connected to interaction with B cells. In addition,

re-construction cannot be mediated without CXCR5 on B cells, showing a strong interplay between B cells and CD21/CD35$^+$ cells in the process of re-construction. The importance of CXCL13/CXCR5 signalling is also known from construction of LN during organogenesis.[10,11] The induction of solitary isolated follicles in the intestine also requires CXCR5/CXCL13 signalling.[26] Therefore and in contrast to the FRC, CXCR5/CXCL13 signalling between B cells and FDC was a prerequisite of the re-establishment and maintenance of the B-cell area. However, it is also possible that LTi cells, which also express CXCR5, contribute to the process of regeneration similar to the process described after LCMV infection.[22]

In conclusion, stromal cells are important key players in maintaining the structure of LN compartments. The FRC act as LN restricted 'self-renewers' by proliferation and reconstructing a network within the T-cell area of the LN. In addition, as they are also positive for CCL19/CCL21 without any expression of these chemokines, T cells can migrate into the T-cell area. This leads to reconstruction of a normal T-cell area. In contrast, CD21/CD35$^+$ cells are recruited from the periphery, but also proliferate to re-establish the B-cell area. The formation of the network and the function of these FDC are dependent on the presence of immigrating B cells and their signalling through CXCR5. Hence, using the *in vivo* LN transplantation model stromal cell populations can be explored in detail. In conclusion, FRC and FDC are able to re-construct and maintain the respective compartment of LN, but they act in different ways.

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Disclosures

The authors have no conflicting financial interests.

References

1 Macpherson AJ, Smith K. Mesenteric lymph nodes at the center of immune anatomy. *J Exp Med* 2006; **203**:497–500.
2 Crivellato E, Vacca A, Ribatti D. Setting the stage: an anatomist's view of the immune system. *Trends Immunol* 2004; **25**:210–7.
3 Willard-Mack CL. Normal structure, function, and histology of lymph nodes. *Toxicol Pathol* 2006; **34**:409–24.
4 Link A, Vogt TK, Favre S *et al.* Fibroblastic reticular cells in lymph nodes regulate the homeostasis of naive T cells. *Nat Immunol* 2007; **8**:1255–65.
5 Luther SA, Tang HL, Hyman PL, Farr AG, Cyster JG. Coexpression of the chemokines ELC and SLC by T zone stromal cells and deletion of the ELC gene in the plt/plt mouse. *Proc Natl Acad Sci USA* 2000; **97**:12694–9.
6 Bajenoff M, Egen JG, Koo LY *et al.* Stromal cell networks regulate lymphocyte entry, migration, and territoriality in lymph nodes. *Immunity* 2006; **25**:989–1001.
7 Mueller SN, Germain RN. Stromal cell contributions to the homeostasis and functionality of the immune system. *Nat Rev Immunol* 2009; **9**:618–29.
8 Forster R, valos-Misslitz AC, Rot A. CCR7 and its ligands: balancing immunity and tolerance. *Nat Rev Immunol* 2008; **8**:362–71.
9 Allen CD, Cyster JG. Follicular dendritic cell networks of primary follicles and germinal centers: phenotype and function. *Semin Immunol* 2008; **20**:14–25.
10 Ansel KM, Ngo VN, Hyman PL *et al.* A chemokine-driven positive feedback loop organizes lymphoid follicles. *Nature* 2000; **406**:309–14.
11 Muller G, Hopken UE, Lipp M. The impact of CCR7 and CXCR5 on lymphoid organ development and systemic immunity. *Immunol Rev* 2003; **195**:117–35.
12 Ngo VN, Korner H, Gunn MD *et al.* Lymphotoxin alpha/beta and tumor necrosis factor are required for stromal cell expression of homing chemokines in B and T cell areas of the spleen. *J Exp Med* 1999; **189**:403–12.
13 Ahrendt M, Hammerschmidt SI, Pabst O, Pabst R, Bode U. Stromal cells confer lymph node-specific properties by shaping a unique microenvironment influencing local immune responses. *J Immunol* 2008; **181**:1898–907.
14 Mori S, Nakano H, Aritomi K, Wang CR, Gunn MD, Kakiuchi T. Mice lacking expression of the chemokines CCL21-ser and CCL19 (plt mice) demonstrate delayed but enhanced T cell immune responses. *J Exp Med* 2001; **193**:207–18.
15 Worbs T, Bode U, Yan S *et al.* Oral tolerance originates in the intestinal immune system and relies on antigen carriage by dendritic cells. *J Exp Med* 2006; **203**:519–27.
16 Bode U, Duda C, Weidner F *et al.* Activated T cells enter rat lymph nodes and Peyer's patches via high endothelial venules: survival by tissue-specific proliferation and preferential exit of CD8$^+$ T cell progeny. *Eur J Immunol* 1999; **29**:1487–95.
17 Buettner M, Pabst R, Bode U. Stromal cell heterogeneity in lymphoid organs. *Trends Immunol* 2010; **31**:80–6.
18 Lammermann T, Sixt M. The microanatomy of T-cell responses. *Immunol Rev* 2008; **221**:26–43.
19 Katakai T, Suto H, Sugai M *et al.* Organizer-like reticular stromal cell layer common to adult secondary lymphoid organs. *J Immunol* 2008; **181**:6189–200.
20 Bajenoff M, Germain RN. B cell follicle development remodels the conduit system and allows soluble antigen delivery to follicular dendritic cells. *Blood* 2009; **114**:4989–97.
21 Hammerschmidt SI, Ahrendt M, Bode U *et al.* Stromal mesenteric lymph node cells are essential for the generation of gut-homing T cells *in vivo*. *J Exp Med* 2008; **205**:2483–90.
22 Scandella E, Bolinger B, Lattmann E *et al.* Restoration of lymphoid organ integrity through the interaction of lymphoid tissue-inducer cells with stroma of the T cell zone. *Nat Immunol* 2008; **9**:667–75.
23 Luther SA, Bidgol A, Hargreaves DC *et al.* Differing activities of homeostatic chemokines CCL19, CCL21, and CXCL12 in lymphocyte and dendritic cell recruitment and lymphoid neogenesis. *J Immunol* 2002; **169**:424–33.
24 Heinzel K, Benz C, Bleul CC. A silent chemokine receptor regulates steady-state leukocyte homing *in vivo*. *Proc Natl Acad Sci USA* 2007; **104**:8421–6.
25 Yang BG, Tanaka T, Jang MH, Bai Z, Hayasaka H, Miyasaka M. Binding of lymphoid chemokines to collagen IV that accumulates in the basal lamina of high endothelial venules: its implications in lymphocyte trafficking. *J Immunol* 2007; **179**:4376–82.
26 Velaga S, Herbrand H, Friedrichsen M *et al.* Chemokine receptor CXCR5 supports solitary intestinal lymphoid tissue formation, B cell homing, and induction of intestinal IgA responses. *J Immunol* 2009; **182**:2610–9.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Several CCL21-positive dendritic cells in the T-cell area of plt-tx.

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