M1 macrophages act as LTβR-independent lymphoid tissue inducer cells during atherosclerosis-related lymphoid neogenesis

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1. Introduction

Newly built lymphoid structures can develop within any non-lymphoid tissues subjected to chronic inflammation. These so-called tertiary lymphoid organs (TLOs) support adaptive immune response induction and maturation. Houtkamp et al.1 have demonstrated that TLOs develop around atherothrombotic arteries. Because TLOs may have an impact on tissue destruction and atheroma progression, elucidating the mechanisms that underlie TLO formation could provide tools to interfere with the generation of local pathological immune effectors. It has been proposed that mechanisms of TLO development can be deduced from the one of the secondary lymphoid organs (SLOs).2,3

During the first step of SLO development, specialized haematopoietic ‘lymphoid tissue inducer’ (LTI) cells interact with stromal cells called ‘lymphoid tissue organizer’ (LTO) cells via α4β1/vascular cell adhesion molecule-1, membrane-bound lymphotoxin (LT)−α1β2/LTβR, and tumour necrosis factor (TNF)−α/TNFRI. The TNFRI induce the nuclear factor kβ (NFκB) pathway involving the translocation of P50/RelA heterodimer to the nucleus. The LTβR also induce the non-classical NFκB pathway that involves the translocation to the nucleus of P52/RelB. This triggers the production of CXCL13, CCL19, and CCL21 chemokines by stromal organizer cells and the

The goal of this study was to characterize the role of inflammatory macrophages in the induction of the vascular smooth muscle cell (VSMC)-mediated formation of aortic tertiary lymphoid organs (TLOs).

Mouse bone marrow-derived M1 macrophages acted as lymphoid tissue inducer cells. Indeed, they expressed high levels of tumour necrosis factor (TNF)-α and membrane-bound lymphotoxin (LT)-α, two inducing cytokines that triggered the expression of the chemokines CCL19, CCL20, and CXCL16, as did M1 supernatant. The blockade of LTβR signalling with LTβR-ig had no effect, whereas that of TNFR1/2 signalling reduced chemokine expression by VSMCs in both wild-type (WT) and LTβR KO mice, demonstrating that LTβR signalling is dispensable for the M1-inducing effect. This effect was corroborated by the development of TLOs observed in LTβR KO → apolipoprotein E knockout (ApoE KO) aortic segments after orthotopic transplantation. Furthermore, treatment of ApoE KO mice with anti-TNF-α antibody decreased the number and incidence of aortic TLOs. Finally, lymphoid nodules composed of T and B cells formed in in vivo-implanted scaffolds seeded with VSMCs previously stimulated ex vivo by M1-conditioned medium.

These results are the first to identify M1 macrophages as inducer cells that trigger the expression of chemokines by VSMCs independently of LTβR signalling. We propose that the dialogue between macrophages and VSMCs—established across the vascular wall—contributes to the formation of aortic TLOs.

Atherosclerosis • Chemokine • Macrophage • Smooth muscle cell • Lymphocyte

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development of high endothelial venules and lymphatics. These molecular events sustain the attraction and trafficking of B, T, and dendritic cells. In the context of atherosclerosis in the apolipoprotein E knockout (ApoE KO) mouse model, Habenicht et al. discovered that vascular smooth muscle cells (VSMCs) in the aortic media act as chemokine-expressing LTα cells in response to TNF–TNFR/LT–LTβR signals. Indeed, VSMCs were able to produce CCL19, CCL21, CXCL13, and CXCL16, which were suspected to promote the recruitment of leucocytes in the adventitia and the generation of TLOs. More recently, a study highlighted the effect of CCL20 on the adventitial recruitment of B cells and the consequent impact on atherosclerotic diseases. These pioneer studies raise the question concerning the identity of the cells supplying the inducing factors to stromal vascular cells and conferring to them an organizer phenotype. In this study, we provide data suggesting that macrophages could be such cells.

Because we noticed in preliminary studies that TLOs could develop at different locations along the aorta, we first setup a new technique to analyse TLO distribution on whole-mount mouse aorta. We confirmed the findings of Grabner et al. and showed that the majority of aged ApoE KO mice develop TLOs in the adventitia of thoracic and abdominal aorta. These TLOs displayed the required features to support the maturation of immune effectors. We next addressed whether macrophages could adopt an LTi profile and found that M1 pro-inflammatory macrophages produced large amounts of TNF-α and LT-α—two key inducing cytokines—and conferred to VSMCs, an organizer phenotype. Such stromal cells were capable to trigger leucocyte recruitment in vivo. To evaluate the signalling pathways involved in the dialogue between macrophages and VSMCs, we used an in vitro approach with blocking antibodies and two in vivo approaches using the orthotopic aortic transposition model and the blockade of TNF-α. Both approaches demonstrated that, in contrast with the canonical formation of SLOs, the LTβR-dependent signalling is not mandatory for the formation of aortic TLOs.

2. Materials and methods

2.1 Animals

LTβR KO (kindly provided by Dr Pfeffer), C57BL/6j, and ApoE KO mice (obtained from the Jackson Laboratory) were bred in our facility. All animals were maintained on a regular chow diet and handled in accordance with European Union directives (86/609/EEC) on the care and use of laboratory animals. For the isolation of macrophages and primary VSMCs, as well as for the analysis of TLOs and atherosclerotic lesions, mice were sacrificed by exsanguination under anaesthesia (intraperitoneal injection of ketamine–HCl 100 mg/kg and xylazine 20 mg/kg). The aorta was dissected for atherosclerotic lesion analysis and for the isolation of primary VSMCs. We harvested the femurs for preparation of macrophages. The investigation was approved by the Animal Ethics Committee of the Institute National de la Santé et de la Recherche Médicale and has therefore been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. Review and approval of the study were also obtained from the Local Animal Ethics Committee (No. B 7518 03). Aortic transplantation was realized as previously described.

2.2 Cell culture

Macrophages were prepared from the femurs of 8-week-old C57BL/6j female mice, as previously described, and were polarized for 15 h either with 100 ng/mL of Lypopolysaccharide (LPS) (Sigma) and 100 U/mL of interferon (IFN)-γ (R&D System) in fetal bovine serum-free medium to obtain inflammatory M1 macrophages or with 25 ng/mL of interleukin-4 (IL-4, R&D System) to obtain alternative M2 macrophages. Primary mouse aortic VSMCs were obtained as described.

2.3 Gene expression analysis

Total RNA was extracted, and mRNA reverse transcription was performed with the iScript reverse transcriptase (Bio-Rad). Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was performed on a CFX 100 (Bio-Rad) cycler using the primers listed in Supplementary material online, Table S1.

2.4 Immunodetection of chemokines

Cell culture supernatants from VSMCs were analysed for CXCL13, CCL19, CCL21, CCL20, and CXCL16 using the BioPlex assay (Bio-Rad), following the instructions of the manufacturer.

2.5 In vivo generation of artificial TLOs

Mouse VSMCs stimulated for 15 h in vitro with M1-conditioned medium were seeded in 40% Matrigel diluted in culture medium (BD Bioscience) and injected subcutaneously into 8-week-old C57BL/6j mice. Each scaffold was loaded with 250 000 VSMCs in 50 μL of Matrigel. The Matrigel was kept in a liquid state at 4°C until the injection, after which the murine internal temperature caused the immediate reticulation of the scaffold. Mice were sacrificed 3 weeks after implantation, and dissected scaffolds were processed for immunohistochemistry.

2.6 Analysis of atherosclerotic lesions

Atherosclerotic lesion size was measured on oil red O-stained frozen transverse sections of LTβR or control C57BL/6j transplanted aortas, as previously described.

2.7 Statistical analysis

Means, SEM, and P-values were calculated using the software Statview. We considered a probability value of <0.05 using a non-parametric test (Mann–Whitney) to be statistically significant.

3. Results

3.1 Characterization of adventitial tertiary lymphoid structures in ApoE KO mice

Whole-mount immunohistochemistry of the entire adventitia from 40-week-old ApoE KO mice (n = 25) revealed that 58% of them developed lymphoid structures that were predominantly found in the thoracic and abdominal parts of the aorta (Figure 1A). These structures, besides being in different maturation stages (as illustrated by the three aggregates shown in Figure 1A), were organized into segregated areas containing either T cells (CD3) or IgD- or IgM-expressing B cells, as evidenced by immunohistochemical analyses performed on abdominal aortic cross-sections (Figure 1B). A high density of cells that stained positive for LYVE-1 and CD31 was detected in an area of TLOs close to the media, indicating the presence of lymphatic and blood vessel networks. ER-TR7-positive cells, which are considered as fibroblastic reticular cells (FRCs) in secondary lymph nodes, were also clearly detected in the same compartment as lymphatic and blood vessels (Figure 1C). Taken together, these immunostaining results revealed that adventitial lymphocyte infiltrates can adopt a TLO compartmental topology.

We could not detect any CD45+CD14+CD3+ prototypic LTi cells in the inflamed arterial wall (data not shown), indicating that other cells must assume the LTi cell role. Given their abundance in human and mouse atherosclerotic arteries, we reasoned that macrophages may...
Figure 1 Adventitial aortic TLOs in hypercholesterolaemic ApoE KO mice. (A) Representative whole-mount IgM, IgD, and CD3 staining on the entire adventitia from 40-week-old ApoE KO mice (n = 25 mice). Dashed boxes represent the lymphoid aggregates that are magnified below (scale bar: 2 mm). Quantitative analysis of TLO incidence is shown in the upper graph. (B) Staining for DAPI (blue), CD3 (purple), IgM (green), and IgD (red) or (C) DAPI (blue), LYVE-1 (red), ER-TR7 (red), and CD31 (red) was performed on transverse cryosections from 57-week-old ApoE KO mouse aortas where elastin autofluorescence was captured (green). Dashed boxes represent areas that are magnified below (scale bar: 50 μm; L: lumen).
take on the role of LTi cells. Indeed, M1 macrophages were present in the lesions of aortas that contained adventitial TLOs (see Supplementary material online, Figure S1). Furthermore, we have previously shown that, according to their evolution stage, atherosclerotic lesions are infiltrated by M1 or M2 macrophages, which have been reported to display many opposing functions; likewise, their capacity to act as LTi cells may also differ. We addressed this issue in the following in vitro experiments.

3.2 M1 inflammatory macrophages as LTi cells

Bone marrow-derived mouse macrophages were polarized towards the M1 or M2 phenotype as previously described. We found that the expression of M1 macrophage markers (iNOS and Arg II), but not of M2 markers (Figure 2A), was associated with robust expression of TNF-α and LT-α (Figure 2B), two key molecules of LTi cells. These data strongly suggest that M1 macrophages can assume the role of LTi cells in atherothrombotic arteries. However, in the context of atherothrombotic arteries, the architecture of the arterial wall, notably the presence of internal and external elastic laminae, results in the almost complete compartmentalization of the cells in each layer (intima, media, and adventitia). Therefore, we reasoned that if macrophages were to establish a dialogue with VSMCs, it had to be based mostly on soluble mediators produced by macrophages and released into the media. We first assessed whether these VSMCs could respond to inducing cytokines.

3.3 VSMCs respond to soluble TNF-α and LT-α

Mouse VSMCs were cultured with recombinant rLT-α and rTNF-α. The levels of chemokine transcripts were measured 15 h after stimulation (Figure 3A). Combined TNF-α and LT-α stimulation induced statistically significant and specific increases in CXCL16, CCL19, and CCL20 transcript levels in VSMCs compared with non-activated cells. The transcript levels for CXCL13 and CCL21 were not affected by the stimulation. The increased levels of CXCL16, CCL19, and CCL20 transcripts in stimulated VSMCs were associated with significant increases in the chemokine concentrations in the supernatants (Figure 3B). We also analysed the individual effect of the two cytokines, and we found that rLT-α and rTNF-α acted in an additive way for the induction of CCL20, while CCL19 and CXCL16 are principally induced by LT-α (see Supplementary material online, Figure S2). These findings demonstrate that VSMCs can be turned into lymphoid organizer cells by inducing cytokines. We next assessed the capacity of M1 macrophage-conditioned medium (M1 CM) to induce chemokine expression in VSMCs.

3.4 M1 inflammatory macrophages confer an LTo profile to VSMCs in an LTβR-independent manner

As observed with recombinant rTNF-α and rLT-α, stimulation of VSMCs from wild-type (WT) mice with M1 CM induced a significant increase in the expression of CXCL16, CCL19, and CCL20 at the mRNA and protein levels (Figure 4A), while stimulation had no effect on CCL21 and CXCL13 expression (data not shown). In contrast, M2 CM had no effect on the expression of any of these chemokines (see Supplementary material online, Figure S3), demonstrating that only M1 macrophages, which produce inducing cytokines, can confer an LTo profile to VSMCs. Importantly, chemokine induction by M1 CM was not due to the macrophage-polarizing medium because LPS and mouse recombinant IFN-γ had only minor effect. To assess the involvement of the LTβR in the stimulation, we added the antagonist LTβR-Ig or its isotype control, and we found that the LTβR-Ig had no effect on the expression and production of chemokines by VSMCs stimulated with M1 CM (Figure 4B).

In contrast, we found that the blockade of TNFR1 and TNFR2 using neutralizing antibodies significantly decreased the levels of chemokine transcripts and the concentrations of chemokine proteins in the supernatant of WT VSMCs stimulated with M1 CM (Figure 4B), demonstrating that TNFR1 and TNFR2 are involved in the VSMC response to inflammatory macrophages.

Figure 2 M1 inflammatory macrophages express inducing cytokines. (A) Macrophages prepared from the femurs of 8-week-old C57BL/6 female mice were cultured for 8 days in a medium containing macrophage colony-stimulating factor-1. They were then polarized towards an M1 [interferon (IFN)-γ + Lypopolysaccharide (LPS)] or M2 (IL-4) phenotype or were left unpolarized (M0). The expression levels of iNOS, Arg I, Arg II, and YM1/2 were determined by RT-qPCR on RNA extracted 15 h after polarization. (B) The expression levels of TNF-α and LT-α were determined by reverse transcription quantitative polymerase chain reaction (RT-qPCR) on RNA extracted from polarized (M1–M2) or non-polarized (M0) macrophages. Data were analysed using the 2−ΔΔCt Pfaffl formula, in which data from polarized macrophages (M1–M2) were compared with Ct values from the non-polarized condition (M0) and normalized to the Ct values of hypoxanthine-guanine phosphoribosyltransferase (HPRT). Data are representative of four independent experiments. *P < 0.05.
We therefore assessed the involvement of TNF-α in vivo. We found that 8 weeks of anti-TNF-α treatment on 15-week-old ApoE mice specifically decreased the percentage of splenic germinal-centre B cells. Furthermore, mice that received the anti-TNF-α treatment presented a decrease in the incidence and number of adventitial TLOs, when compared with control mice (Figure 4C).

Intriguingly, these results strongly suggest that the canonical LTβR signalling may be dispensable for the VSMC response to M1 CM. To directly address this issue, we performed the same experiment as above with VSMCs prepared from the aorta of LTβR KO mice. Our results indicate that M1 CM stimulation of VSMCs from LTβR KO mice induced increases in the expression levels of CXCL16, CCL19, and CCL20 (mRNA and protein; Figure 4D). Interestingly, the concentrations of these three chemokines were sensibly higher in the supernatants of stimulated VSMCs from LTβR KO mice compared with those from WT mice. This difference could be explained by compensatory increases in the expression of TNF receptors in the absence of LTβR because we also detected increased transcript levels for TNFR1 and TNFR2 in LTβR KO VSMCs compared with WT VSMCs (see Supplementary material online, Figure S4), which endow VSMC with higher sensitivity to M1 CM.

To confirm that LTβR signalling is dispensable for the formation of abdominal aortic TLOs in vivo, we used a mouse model of aortic transplantation in which the infrarenal abdominal aortic segment from an LTβR KO mouse is placed in an orthotopic position in an ApoE KO mouse. We used C57BL/6J aortic segments as control. Donor and recipient mice were histocompatible. This model was preferred to an experimental approach based on LTβR inhibition with an injected soluble antagonist18 or to a breeding strategy between LTβR KO and ApoE KO mice, as disruption (LTβR inhibition) or a lack of peripheral lymph node development (LTβR KO) in ApoE KO mice would have been a confounding factor to interpret the absence of LTβR signalling for TLO formation. Instead, in the orthotopic aortic transplantation of LTβR KO--→ApoE KO mice in which the immune system is integral, we can study the effect of a local disruption of LTβR signalling on vascular stromal cells. LTβR KO--→ApoE KO transplanted mice were sacrificed 4 months after surgery, and immunohistochemical analyses of transplanted aortic segment cross-sections were performed. Despite the absence of LTβR (Figure 5A), transplanted aortic segments systematically contained adventitial lymphoid structures (Figure S8) that, in both conditions, presented characteristics of highly mature structures, with T and B cell areas, CD31+ high endothelial venules, LYVE-1+...
lymphatic endothelium, and ER-TR7\textsuperscript{+} FRC-like cells. Quantitative analyses showed that atherosclerotic lesions were of similar size in C57BL/6J and LT\textsuperscript{bR} transplanted aortas (Figure 5C). Furthermore, we found no difference in terms of B and T cell area and atherosclerotic lesion structure between the two groups (see Supplementary material online, Figure S5).

Taken together, these data demonstrate that LT\textsuperscript{bR} signalling is dispensable for the formation of abdominal aortic adventitial TLOs.

### 3.5 VSMCs can trigger the ectopic generation of TLO structures \textit{in vivo}

To definitively validate the capacity of M1 macrophages to act as LTi cells on VSMCs and to test whether these stromal cells can act as LTi cells, we tested whether the increases in CXCL16, CCL19, and CCL20 expression observed \textit{in vitro} in M1 CM-stimulated VSMCs could effectively be responsible for the chemotraction of immune cells and their organization into TLOs \textit{in vivo}. To this end, we seeded VSMCs from C57BL/6J

![Figure 4](image-url)

**Figure 4** M1 macrophages confer an LTi phenotype to VSMCs in an LT\textsuperscript{bR}-independent manner. Primary aortic VSMCs from C57BL/6J (A and B) or LT\textsuperscript{bR} KO (D) mice were stimulated (LPS M1 CM or LPS + IFN-\gamma) or not (NA) for 15 h with M1 CM with (+) or without (−) TNFR1, TNFR2, or TNFR1/2-blocking antibodies or LT\textsuperscript{bR}-Ig. The expression levels of CXCL16, CCL20, and CCL19 were determined by RT-qPCR on extracted RNA. Data were analysed using the 2\textsuperscript{−ΔΔCt} Pfaffl formula, \cite{Pfaffl1996} in which \( C_t \) values from stimulated VSMCs were compared with those from unstimulated cells and normalized to the \( C_t \) values of HPRT. The concentrations of CXCL16, CCL20, and CCL19 in the supernatants of VSMCs were determined using cytometric bead assays. Data are representative of four independent experiments. (C) Fifteen-week-old female ApoE KO mice were injected intraperitoneally and weekly with 350 \( \mu \)g anti-TNF-\( \alpha \) antibody or with the same amount of isotype control. After 8 weeks of treatment, adventitial TLO incidence and number were assessed on whole-mount aorta and splenic naïve (B220\textsuperscript{+}, CD21\textsuperscript{−}, and CD23\textsuperscript{−}) or germinal-centre (B220\textsuperscript{+}, CD95\textsuperscript{+}, and IgM\textsuperscript{low}) B cells (B220) were analysed by flow cytometry. *\( P \leq 0.05 \).
or LTβR KO mice within Matrigel scaffolds that were then injected subcutaneously in mice. Prior to seeding, VSMCs had been stimulated or not ex vivo with M1 CM. We found that, independently of the VSMC genotype, the incidence of aggregate formation was increased in Matrigel scaffolds containing M1 CM-stimulated VSMCs compared with scaffolds seeded with unstimulated cells (Figure 6A and B). Importantly, these infiltrates were composed of CD3⁺ T and B220⁺ B lymphocytes (Figure 6C). In addition, VSMCs still present in the scaffold [α-smooth muscle actin (SMA)-positive cells] expressed CXCL16, CCL19, and CCL20 (Figure 6D), while CCL21 and CXCL13 were not detected, corroborating our previous RT-qPCR results obtained on cultured cells. Taken together, these data confirm—in an in vivo model—that M1 macrophages act as LTi cells on stromal VSMCs, which, in turn, trigger the recruitment of lymphoid cells and their organization into TLO structures.

4. Discussion

In contrast to SLOs that arise during development at predetermined locations, the formation of TLOs can occur in adults at ectopic sites in any tissue in the context of persistent inflammatory disorders, such as autoimmune diseases, cancers, and atherothrombotic diseases. Here, we have developed a new immunohistochemical technique involving whole-mount staining of the adventitia from ApoE KO mice, which allowed us to study the distribution of the lymphoid aggregates all along the aorta. These structures were defined as TLOs because they were composed of B cell follicles surrounded by T cells, a prototypic organization reported for ectopic germinal centres. IgM and IgD staining revealed the presence of two subsets of B cells with presumably different maturation states. Moreover, these TLOs included blood

Figure 5 Transplanted LTβR KO aortas develop organized TLOs. LTβR KO or C57BL/6 j aortic segments were transplanted in an orthotopic position by end-to-end anastomosis into ApoE KO mice (n = 5 of 5, scale bar: 20 μm). (A) LTβR (red), DAPI (blue), and autofluorescent elastin (green) staining on transversal cryosections of LTβR KO or C57BL/6 j aortic segments explanted from ApoE KO mice 4 months after transplantation. (B) DAPI (blue, scale bar: 100 μm) immunostaining was performed on transverse cryosections of LTβR KO or C57BL/6 j aortic segments explanted from ApoE KO mice 4 months after transplantation. Dashed boxes represent areas of TLOs that are magnified below for DAPI (blue), CD3 (purple), IgM (green), IgD (red), ER-TR7 (cyan), CD31 (red), and LYVE-1 (purple) staining (scale bars: 50 μm). (C) Atherosclerotic lesion size in WT and LTβR KO aortic segments explanted from ApoE KO mice 4 months after transplantation.
vessels, lymphatic networks, and FRC-like cells, suggesting that these aggregates are proper structures to induce and maintain local immune responses. These adventitial blood and lymphatic networks are essential for the recruitment and drainage of immune effectors.24–27 Interestingly, the TLOs were polarized towards the media, suggesting that effectors inside the aortic wall may be involved in the lymphangiogenesis and angiogenesis associated with the formation of adventitial TLOs. In this respect, it is important to note that human VSMCs are able to trigger intramural angiogenesis through the production of vascular endothelial growth factor A.28 The lymphangiogenesis that is associated with lymphoid neogenesis remains poorly understood. Several studies have demonstrated that, in the context of persistent inflammation, macrophages were able to induce the formation of lymphatic vessels.29,30

In the inflamed aortas of ApoE KO mice, Grabner et al.8 performed a detailed characterization of TLOs and showed that VSMCs in the media expressed the chemokines such as CXCL13, CCL19, CCL21, and CXCL16, and were hence believed to trigger the recruitment of leucocytes in the adventitia. Here, we sought to identify the cellular and molecular factors that confer an organizer (LTo) potential to VSMCs and to directly address whether such organizer VSMCs were able to chemoattract leucocytes in an in vivo model. We hypothesized that macrophages could take on the role of LTi cells in the context of atherothrombosis because they are abundant in inflamed human and mouse arteries, and their role in the progression of disease is well established.31 In addition, we have previously reported their extreme plasticity, which is illustrated by the switch from an initial reparative M2 phenotype to a more inflammatory M1 phenotype during the progression of atherosclerosis.12

Our data suggest that M1 macrophages are relevant LTi candidates because they express markedly higher LT-α and TNF-α levels than non-polarized M0 or reparative M2 macrophages. However, in the context of vascular lymphoid neogenesis, the architecture of the arterial wall prevents macrophages infiltrated within the intimal lesions from directly interacting with medial VSMCs. Indeed, the internal elastic lamina represents an insuperable obstacle for most leucocytes, which are rarely found within the arterial medial layer except in extreme situations where the extracellular matrix is attacked. In contrast, soluble mediators generated within the intima are forced by the radial hydrolytic conductance to move outward into the media via the fenestrations of the elastic laminae.32 We thus hypothesized that intimal macrophages, instead of acting through ‘classical’ cell–cell interactions that characterize LTo induction, assume an LTi role remotely, via soluble factors, thereby endowing VSMCs with LTo functions. To reproduce the effect of soluble mediators expressed by intimal macrophages, we evaluated the effect of M1 CM on VSMC chemokine expression. M1 CM

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**Figure 6** VSMCs can trigger the ectopic generation of TLO structures in vivo. Matrigel scaffolds were seeded with murine VSMCs from LTβR KO or WT mice stimulated with M1 CM (VSMC<sup>LTo</sup>) or unstimulated (NA). (A) TLO incidence in Matrigel scaffolds (n = 10 mice per condition). (B) Representative haematoxylin-eosin (scale bar: 0.5 mm) staining on Matrigel scaffold seeded with M1 CM-stimulated VSMC from WT mice, in which dashed boxes 1 and 2 represent areas where immunostaining in (C) and (D) panels, respectively, were magnified. (C) DAPI (blue), B220 (red), and CD3 (green) (scale bar: 20 μm) and (D) DAPI (blue), α-SMA (green), CCL19 (red), CXCL16 (red), and CCL20 (red; scale bar: 10 μm) staining were performed on explanted scaffolds 20 days after subcutaneous implantation in mice.
significantly increased the expression of CCL19, CCL20, and CXCL16 by VSMCs. This effect might be the result of the increased expression of the inducing cytokines by M1 macrophages, as suggested by our results demonstrating that soluble recombinant LT-α and TNF-α induced the same expression profile in VSMCs. TFN-α and LTs are members of the TNF ligand and receptor superfamily that mediate a multitude of inflammatory responses. While TNF-α and soluble homotrimeric LT-α3 (also called TFN-β) have been reported to interact with TNFR1 and TNFR2, the membrane-bound heterotrimeric LT-α1β2 binds to LTβR. The fact that soluble mediators within CM from M1 macrophages could confer an LT0 phenotype to VSMCs indicated that this effect was therefore LTβR-independent and relied upon TNFR1/2 signalling. To confirm this hypothesis, we first tested the effect of the antagonist LTβR-Ig on WT VSMCs, showing no difference in the induction of chemokine expression and production. We then tested the effect of TNFR1/2-neutralizing antibodies on VSMCs from WT or LTβR KO mice. Our data clearly show that M1 CM induced an increase in chemokine expression in VSMCs derived from either LTβR KO or WT mice. Furthermore, the blockade of TNFR1/2 signalling significantly reduced CXCL16, CCL19, and CCL20 production by stimulated VSMCs from both types of mice. In most cases, the two TNFR-neutralizing antibodies displayed an additive inhibitory effect on chemokine expression by VSMCs.

Interestingly, we observed that the chemokine production was higher in VSMCs from LTβR KO mice compared with WT VSMCs. This difference could be explained by compensatory increases in the expression of TNF receptors in VSMCs from LTβR KO mice, which likely render them more responsive to TNF-α and LT-α3. Transcriptional activities and protein concentration were co-modulated for most of the tested chemokines. However, high transcriptional activity was not strictly correlated with protein concentration in every case. For instance, the CCL19 mRNA level was dramatically increased upon stimulation, but was accompanied by only a modest increase in protein level (Figures 3 and 4). This may be due to a time delay between mRNA transcription and protein translation, post-transcriptional regulation of CCL19 mRNA that would prevent its translation, and/or to the instability of the CCL19 protein leading to an underestimation of the amount produced.

The results obtained in vitro were corroborated by those collected in the in vivo anti-TNF-α and aortic transposition experiments. Indeed, the treatment of ApoE KO mice with anti-TNF-α decreased the number and incidence of adventitial TLOs. Furthermore, we demonstrated the presence of highly mature adventitial aortic TLOs in control WT and also in LTβR KO segments that had been transplanted into ApoE KO mice. Moreover, the selective staining for LTβR in cells surrounding the LTβR KO donor aorta clearly indicates that the cells from the recipient mice did not migrate along the LTβR KO aortic segment and therefore that the stromal cells in the associated TLOs were of donor origin. We can hence deduce that recipient cells did not compensate for the lack of LTβR signalling in the aortic graft. Taken together, these findings demonstrate that, in the context of atherosclerosis-associated lymphoid neogenesis, LTβR signalling is dispensable in VSMCs.

To definitively validate these observations, we used collagen scaffolds seeded with VSMCs from WT or LTβR KO mice that had been stimulated or not ex vivo with M1 CM. These scaffolds were then implanted subcutaneously in mice. Three weeks after implantation, we found that scaffolds seeded with M1 CM-stimulated VSMCs contained lymphoid cell aggregates, independently of the VSMC genotype. The lymphoid cells that composed these aggregates were CD3+ T and B220+ B cells and were distributed quite randomly within the aggregate, indicating an early maturation stage of these aggregates. Immunohistochemical analysis revealed that SMA-positive VSMCs surrounding the aggregates expressed CCL19, CCL20, and CXCL16. Notably, the inducing signal mediated by M1 macrophages did not affect the expression of CCL21 and CXCL13. This suggests that additional local mediators within the inflamed arterial wall may be required to endow VSMCs with a full LT0 phenotype.

Taken together, our data show that LTβR is dispensable for the formation of VSMC-mediated aortic TLOs. This may appear discordant with previous studies showing that LTβR is crucial for the formation or the maintenance of TLOs, but where the experimental design involved the induced expression of CXCL13. In our experimental approach, VSMCs did not produce CXCL13 but other chemokines that were sufficient to trigger the maturation and maintenance of lymphoid aggregates.

In theory, macrophage depletion could conclusively demonstrate that they trigger the organizer phenotype of VSMC in vivo. This could be achieved by clodronate treatment or bone marrow transplantation from colony stimulating factor receptor 1 KO mice. However, this would not solely prevent macrophages from acting on VSMCs, as it would also impair lesion development and thereby TLO formation, precluding the interpretation of such an experiment.

In summary, for the first time, we show that classical LTi cells might not be mandatory for the development of TLOs, a role that can be assumed by macrophages. We propose that, in the context of atherosclerosis, soluble mediators produced by intimal M1 macrophages may activate medial VSMCs through their TNF receptors independently of LTβR signalling. Our findings support the hypothesis that radial hydraulic conductance can convey information from the intima towards the adventitia: intimal macrophages could transmit LTi-inducing signals to medial VSMCs, which, in turn, would orchestrate chemotraction and lymphoid neogenesis in the adventitia. While we did not provide the demonstration that the macrophage–VSMC interaction is the founding element for the formation of aortic TLOs, we nevertheless demonstrate that this interaction is sufficient to confer VSMC with LT0 capacities. To our knowledge, this is the first demonstration, at a functional level, that TLOs can arise as a result of this dialogue.

**Supplementary material**

Supplementary material is available at Cardiovascular Research online.

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**References**


sharing in donor-specific and non-donor specific alloantibodies generation. Transpl

rejection triggers the development of an aggressive intragraft immune response through


alpha/beta and tumor necrosis factor are required for stromal cell expression of

6. Wore CF, VanArsdale TL, Crowe PD, Browning JL. The ligands and receptors of the lym-

receptor signaling promotes tertiary lymphoid organogenesis in the aorta adventitia

smooth muscle cells differentiate into lymphoid tissue organizer-like cells on combined
tumor necrosis factor receptor-I/lymphotoxin beta receptor NF-kappaB signaling.


10. Thaunat O, Field AC, Dai J, Louedec L, Patey N, Bloch MF et al. Lymphoid neogenesis in
chronic rejection: evidence for a local humoral alloimmune response. Proc Natl Acad Sci

11. Thaunat O, Field AC, Dai J, Louedec L, Patey N, Bloch MF et al. Lymphoid neogenesis in
chronic rejection: evidence for a local humoral alloimmune response. Proc Natl Acad Sci

12. Thaunat O, Field AC, Dai J, Louedec L, Patey N, Bloch MF et al. Lymphoid neogenesis in
chronic rejection: evidence for a local humoral alloimmune response. Proc Natl Acad Sci

chronic rejection: evidence for a local humoral alloimmune response. Proc Natl Acad Sci

chronic rejection: evidence for a local humoral alloimmune response. Proc Natl Acad Sci

15. Thaunat O, Field AC, Dai J, Louedec L, Patey N, Bloch MF et al. Lymphoid neogenesis in
chronic rejection: evidence for a local humoral alloimmune response. Proc Natl Acad Sci

chronic rejection: evidence for a local humoral alloimmune response. Proc Natl Acad Sci

17. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR.
Nucl Acids Res 2001;29:44.

necrosis factor functions to maintain splenic architecture and humoral responsiveness in

transferase is expressed in rheumatoid arthritis synovium and is induced by
lymphotoxin-alpha/beta and TNF-alpha in cultured endothelial cells. BMC Immunol

20. Stott DJ, Hiepe F, Hummel M, Steinhauser G, Berek C. Antigen-driven clonal prolifer-
ation of B cells within the target tissue of an autoimmune disease. The salivary glands

21. Steere AC, Duray PH, Butcher EC. Spirochetal antigens and lymphoid cell surface
markers in Lyme synovitis. Comparison with rheumatoid synovium and tonsillar lymph-

22. Baddoura FK, Nasr IW, Wrobel B, Li Q, Ruddle NH, Laklits PG. Lymphoid neogenesis in

23. Drayton DL, Liao S, Mounzer RH, Ruddle NH. Lymphoid organ development: from

24. Miyasaki M, Tanaka T. Lymphocyte trafficking across high endothelial venules: dogmas

25. Gallatin WM, Weissman IL, Butcher EC. A cell-surface molecule involved in organ-

26. Randolph GJ, Angeli V, Swartz MA. Dendritic-cell trafficking to lymph nodes through

27. Sasaki H, Moore AM, Brown MJ, Hwang ST. Cutting edge: secondary lymphoid-tissue-ten-
molecule (SLC) and CC chemokine receptor 7 (CCR7) participate in the emigration
pathway of mature dendritic cells from the skin to regional lymph nodes. J Immunol
1999;162:2472–2475.

Early atheroma-derived agonists of peroxisome proliferator-activated receptor-gamma
trigger intramedial angiogenesis in a smooth muscle cell-dependent manner. Circ Res

29. Ligresti G, Aplin AC, Zarrà P, Morishita A, Nicosia RF. Macrophage-derived tumor necro-
sis factor-alpha is an early component of the molecular cascade leading to angiogenesis


32. Lacolley P, Regnault V, Nicoletti A, Li Z, Michel JB. The vascular smooth muscle cell in
experimental atherosclerosis: role of integrins and other surface molecules in atheroma


34. Luther SA, Lopez T, Bai W, Hanahan D, Cyster JG. BLC expression in pancreatic islets
causes B cell recruitment and lymphoid-dependent lymphoid neogenesis. Immunity

35. MacKay F, Majews GR, Lawton P, Hochman JS. Lymphotoxin but not tumor
necrosis factor functions to maintain splenic architecture and humoral responsiveness in

necrosis factor functions to maintain splenic architecture and humoral responsiveness in