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Lymphoid neogenesis in vascular chronic inflammation

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In contrast to secondary lymphoid organs (SLOs) that arise during development at predetermined locations, the formation of tertiary lymphoid organs (TLOs) can occur in adults at ectopic sites in any tissue in the context of persistent inflammatory disorders, such as autoimmune diseases, cancer, and organ transplantation(1-3). The molecular mechanisms underlying the organization of chronic inflammatory infiltrates into ectopic lymphoid tissue recapitulatesome of those involved in lymphoid organogenesisduring development(4, 5) and hence this process hasbeen referred to as lymphoid neogenesis. Beyond anatomical similarities with SLOs, ectopic lymphoid tissues are fully functional and support the development of local adaptive immune responses, including the priming of naive lymphocytes (6), generation of memory subsets, and germinal center reactions (clonal expansions, somatic hypermutations, immunoglobulin class switching and antibody production), which are suspected to contribute to the exacerbation ofchronic inflammatory diseases (7, 8).

1/ TLOs in rejected organs

We have undertaken several studies in the context of alloimmunity and have demonstrated 1/ that the graft is not only the target of the alloimmune response but also a site where this response actually develops, so as to optimize the communication between the targeted tissue and the immune effectors(9), 2/ that TLOs provide survival signal to B-cells, allowing them to escape rituximab-induced apoptosis, thereby thwarting therapeutic efficiency (10), 3/ that anti-MHC humoral response is more intense and more diverse in TLOs [the abnormal activation of CD4+ T cells promotes the development of an exaggerated pathogenic immune humoral response in TLOs due to a defective immune regulation(11) and the intragraft microenvironment interferes with peripheral deletion of autoreactive immature B cells that, in turn, produce antibodies against intracellular autoantigens

(12)], 4/ that intragraft humoral immune response appears uncoupled from the systemic response and that TLO formation recapitulate organogenesis of SLOs (13).

While these data demonstrate that chronic rejection isassociated with the development of lymphoid nodularinfiltrates within rejected organs, evidence for the involvement of these lymphoid structures in the rejection process came from a model of rat aortic interposition model where lymphoid nodular infiltrates was evidenced in the adventitia of the chronically rejected aorticgrafts (3). We could demonstrate that nodular lymphoidstructures were functional ectopic germinal centers thatparticipate in the rejection process of the grafted organ. In addition, this showed that the vascular stroma was sufficient to promote and support lymphoid neogenesis.

2/ TLOs in atherothrombosis

Interestingly, TLOs are also present in the context of atherothrombosis. Indeed, TLOs were detected in the adventitia of human atherosclerotic arteries as early as the 1950s(14), a process that has been recently revisited(15-18). We couldalso characterize adventitial lymphoid aggregates distributed all along the aortic segment in atherosclerosis-prone ApoE KO mice. These structures were defined as TLOs because they are composed of B cell follicles surrounded by T cells, a prototypic organization reported for ectopic germinal centers(4). IgM and IgD staining revealed the presence of two subsets of B cells with different maturation states. Moreover, these TLOs included blood 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(19-22). 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 VEGF-A(23).

3/ Mechanisms of lymphoid neogenesis in the aorta

A detailed characterization of TLOs in ApoE KO mouse aortas showed that vascular smooth muscle cells (VSMCs) in the media express the chemokines CXCL13, CCL19, CCL21, and CXCL16, triggering the recruitment of leukocytes to the adventitia(16). This raised the question of the identity of the cellular and molecular factors that confer an organizer (LTo) potential upon VSMCs. We hypothesized that macrophages could act as Lymphoid Tissue inducer (LTi) cells in diseased arteries.

Indeed, macrophages are key players in host defense and tissue homeostasis and are heterogeneous and plastic cells that can mount different programs of polarized activation according to microenvironmental cues (24). M1-polarized macrophages are considered as pro-inflammatory cells that produce high levels of effectors, such as the cytokine interleukin-1β, while M2-polarized macrophages are rather reparative cells, promoting the synthesis of extracellular matrix components. In the context of advanced atheroma, plaque-infiltrated macrophages have an M1 pro-inflammatory profile(25, 26).

Our data suggest that M1 inflammatory macrophages are relevant LTi candidates because they express high LT-α and TNF-α levels. However, instead of acting through “classical” cell-cell interactions that characterize LTo induction, intimal macrophages assume an LTi role remotely, via soluble factors, thus endowing VSMCs with LTo functions. We further showed that the effect of M1 macrophages on VSMCs involved TNFR1/2 signaling and was LTβR-independent

The results obtained *in vitro*were corroborated by those collected in an*in vivo* aortic transposition model. Indeed, we demonstrated the presence of adventitial aortic TLOs in LTβR KO segments that had been transplanted into ApoE KO mice. Together, these findings demonstrate that, in the context of atherosclerosis-associated lymphoid neogenesis, LTβR signaling is dispensable.

To definitively validate these observations, we used collagen scaffolds seeded with VSMCs that had been stimulated *ex vivo* with M1 conditioned medium (CM) or with control media. 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, which were not detected in scaffolds containing unstimulated VSMCs. The lymphoid cells that composed these aggregates were $CD3^+$ T and $B220^+$ B cells. SMA-positive VSMCs surrounding the aggregates expressed CCL19, CCL20, and CXCL16.

In summary, for the first time, our most recent data show that classical LTi cells might not be mandatory for the development of TLOs, a role that can be assumed by macrophages. Indeed, in the context of atherosclerosis, soluble mediators produced by intimal M1 macrophages activate medial VSMCs through their TNF receptors independently of LTβR signaling. These findings support the hypothesis that radial hydraulic conductance conveys information from the intima toward the adventitia in the following manner: intimal macrophages transmit LTi-inducing signals to medial VSMCs, which, in turn, orchestrate chemoattraction and lymphoid neogenesis in the adventitia.

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