

## Adipocytes orchestrate the formation of tertiary lymphoid organs in the creeping fat of Crohn's disease affected mesentery



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### ABSTRACT

The formation of tertiary lymphoid organs (TLOs) is orchestrated by the stromal cells of tissues chronically submitted to inflammatory stimuli, in order to uphold specific adaptive immune responses. We have recently shown that the smooth muscle cells of the arterial wall orchestrate the formation of the TLOs associated with atherosclerosis in response to the local release of TNF- $\alpha$ . Observational studies have recently documented the presence of structures resembling TLOs the creeping fat that develops in the mesentery of patients with Crohn's disease (CD), an inflammatory condition combining a complex and as yet not elucidated infectious and auto-immune responses.

We have performed a comprehensive analysis of the TLO structures in order to decipher the mechanism leading to their formation in the mesentery of CD patients, and assessed the effect of infectious and/or inflammatory inducers on the potential TLO-organizer functions of adipocytes.

Quantitative analysis showed that both T and B memory cells, as well as plasma cells, are enriched in the CD-affected mesentery, as compared with tissue from control subjects. Immunohistochemistry revealed that these cells are concentrated within the creeping fat of CD patients, in the vicinity of transmural lesions; that T and B cells are compartmentalized in clearly distinct areas; that they are supplied by post-capillary high endothelial venules and drained by lymphatic vessels indicating that these nodules are fully mature TLOs.

Organ culture showed that mesenteric tissue samples from CD patients contained greater amounts of adipocyte-derived chemokines and the use of the conditioned medium from these cultures in functional assays was able to actively recruit T and B lymphocytes. Finally, the production of chemokines involved in TLO formation by 3T3-L1 adipocytes was directly elicited by a combination of TNF- $\alpha$  and LPS *in vitro*.

We therefore propose a mechanism in which mesenteric adipocyte, through their production of key chemokines in response to inflammatory/bacterial stimuli, may orchestrate the formation of functional TLOs developing in CD-affected mesentery.

### 1. Introduction

Crohn's disease (CD) is a chronic inflammatory disease of the digestive tract, characterized by destructive lesions of variable topography, depth, and size. Interestingly, CD-related intestinal lesions are characterized by a hypertrophy of the adjoining mesenteric adipose tissue, a process called "sclerolipomatosis" or "creeping fat" [1]. This

process is extremely specific to CD and is not observed in other chronic forms of enteritis. The extent of creeping fat correlates closely with transmural inflammation, fibrosis, muscularization, and stricture formation [2] and is no longer considered a passive marker of intestinal inflammation, but rather a pathogenic process supporting the release of pro-adipokines [3,4] and local amplification of the inflammatory response in response to recurrent intestinal ulcerations ineluctably

**Abbreviations:** CD, "Crohn's Disease"; LPS, "Lipopolysaccharide"; LTo, "Lymphoid Tissue organizer"; LTi, "Lymphoid Tissue inducer"; SLO, "Secondary Lymphoid Organ"; TLO, "Tertiary Lymphoid Organ"; TNF, "Tumor Necrosis Factor"; VSMC, "Vascular Smooth Muscle Cell"

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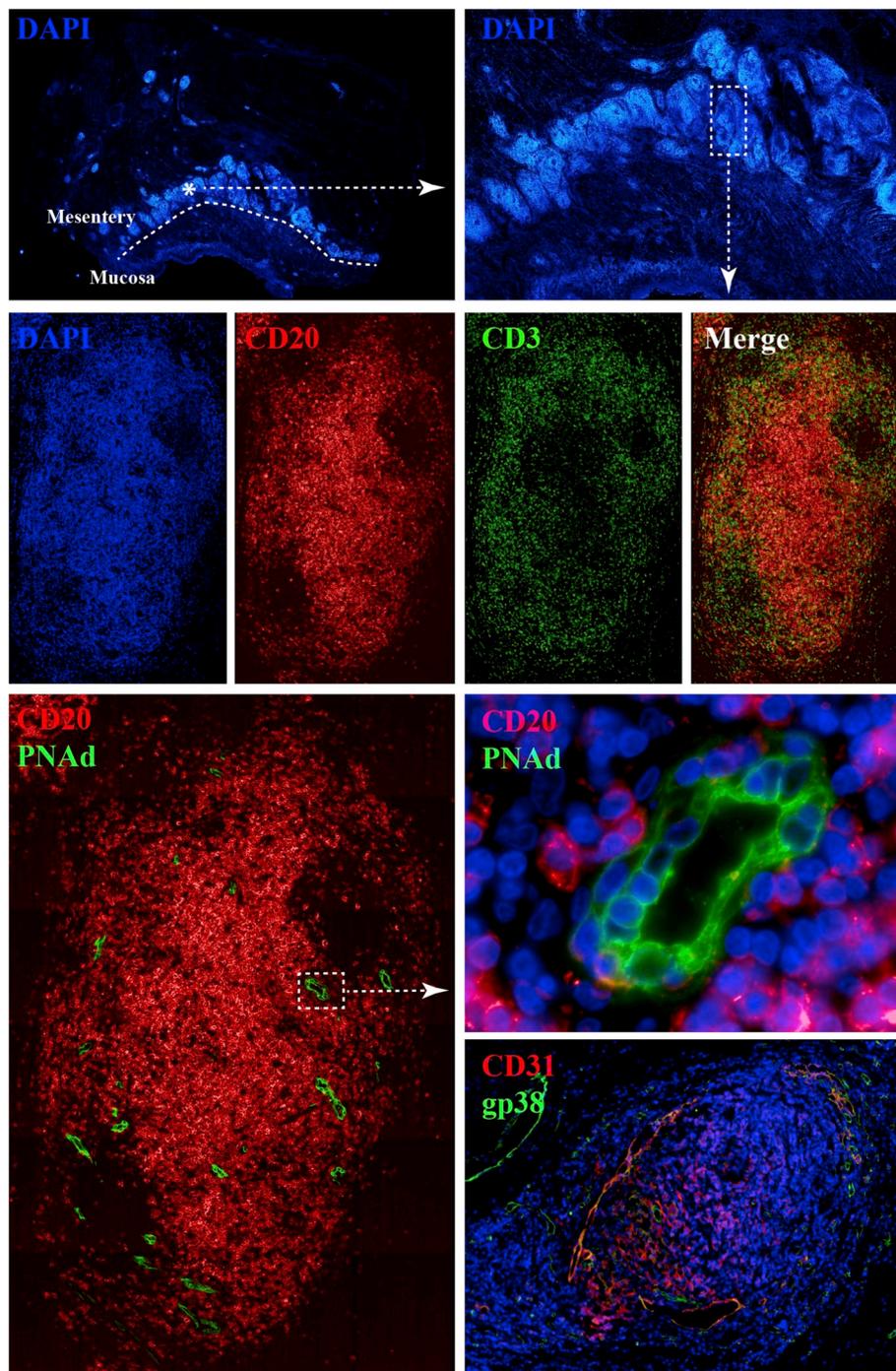
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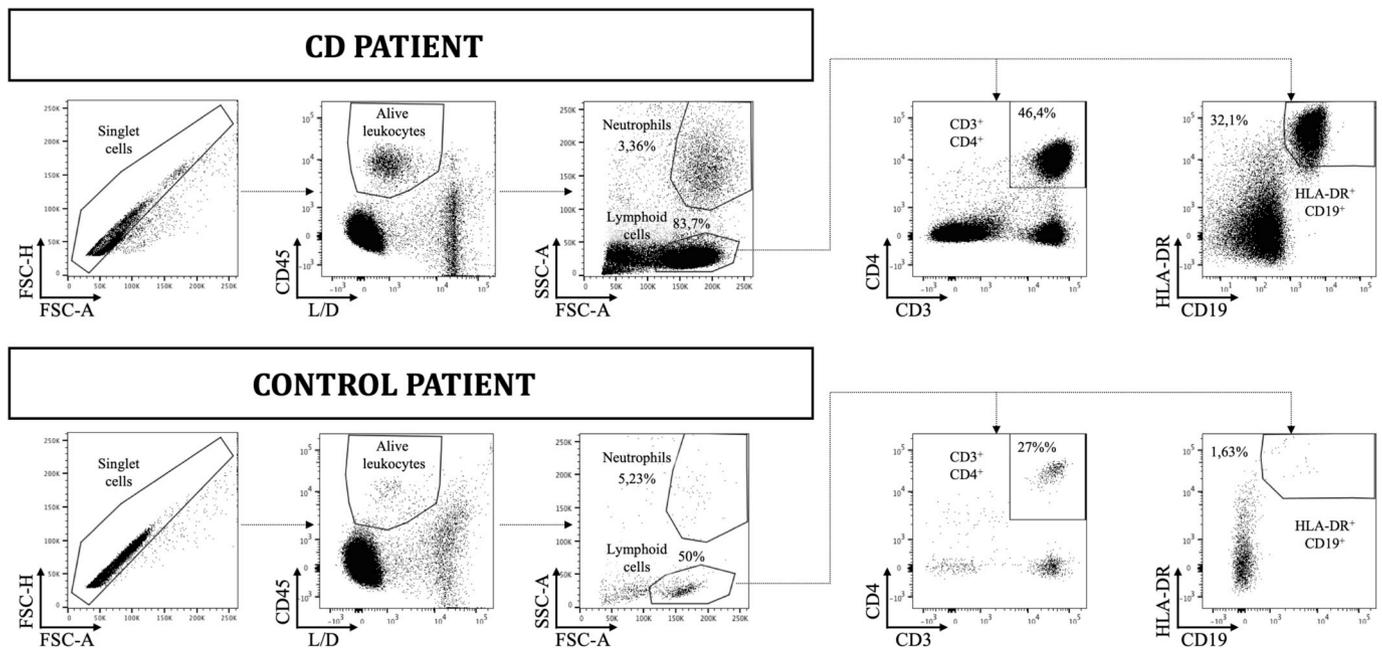
**Fig. 1.** B cell follicles develop in the mesentery of human CD. Representative staining of DAPI, CD20, CD3, PNAd, and gp38 performed on human intestinal wall cross-sections from CD patients. The asterisk and the dashed boxes represent the magnified area, indicated by the arrow.

accompanied by bacterial translocation [5].

Recent reports show that lymphoid nodules resembling tertiary lymphoid organs or TLOs are found impinged on lymphatic collecting vessels that enter and exit lymph nodes in the CD-affected mesentery [6,7]. We had previously shown that TLOs develop in the medial layer and adventitia of chronically inflamed arteries, likely due to the lymphatic drainage blockade [8]. The formation of TLOs is orchestrated by the stromal cells of tissues chronically submitted to inflammatory stimuli and, in the case of atherosclerosis, we showed that the organizer role can be undertaken by arterial smooth muscle cells, in response to the local release of TNF- $\alpha$ . For triggering the formation of CD-associated TLOs, it is plausible that pathogen-derived stimuli might act in

concert with TNF- $\alpha$  as blockade of the latter, alone, is not sufficient to suppress their formation in an experimental mouse model of CD.

In the present study, we demonstrate that the addition of a pathogen-derived stimulus, such as bacterial LPS to TNF- $\alpha$ , is able to confer adipocytes with a phenotype that resembles the one of “lymphoid tissue organizer cells”, i.e. the stromal cells capable of orchestrating the formation of organized lymphoid structures. Our findings offer an explanation for the preferential development of striking fully mature TLOs within the hypertrophic, “creeping” adipose tissue that characterizes CD mesentery.



**Fig. 2.** Increased inflammatory infiltrate in the mesentery of CD. Representative diagrams showing flow cytometry analysis of CD3<sup>+</sup>CD4<sup>+</sup> T lymphocytes, CD19<sup>+</sup>HLA-DR<sup>+</sup> B lymphocytes and neutrophils among CD45<sup>+</sup> Live/Dead<sup>-</sup> alive leukocytes, performed on mesenteric digests from either CD patients or control patients (right colic adenocarcinoma).

## 2. Material and methods

### 2.1. Selection of patients and tissue collection

This prospective and monocentric cohort study was performed on resection specimens of ileum from patients (n = 8) operated for CD or from controls (n = 6) operated for right colon adenocarcinoma. All patients provided informed written consent, and the protocol was approved by a French ethics committee (Comité d'Évaluation de l'Éthique des projets de Recherche Biomédicale Paris Nord; N° 2018-014).

### 2.2. Cell and tissue analyses

Human tissues were characterized by immunohistochemistry. Cell suspensions were analyzed by flow cytometry. Please refer to the online-only Supplementary Data for the detailed methods.

### 2.3. Chemotaxis experiment

Chemotaxis assays were performed using polycarbonate filter with 5 μm pores (Neuro Probe). Please refer to the online-only Supplementary Data for the detailed methods.

### 2.4. Immunodetection of chemokines

Chemokine content was analyzed using the BioPlex® assay (Bio-Rad), as described in the online-only Supplementary Data.

### 2.5. Gene expression analysis

Total RNA was analyzed by real-time polymerase chain reaction, as described in the online-only Supplementary Data.

### 2.6. Statistical analysis

The means, SEMs and p values were calculated using the software JMP®. The differences between conditions were considered statistically significant for the probability value < 0.05 using Student t-tests and

Mann-Whitney non-parametric tests, as appropriate.

## 3. Results

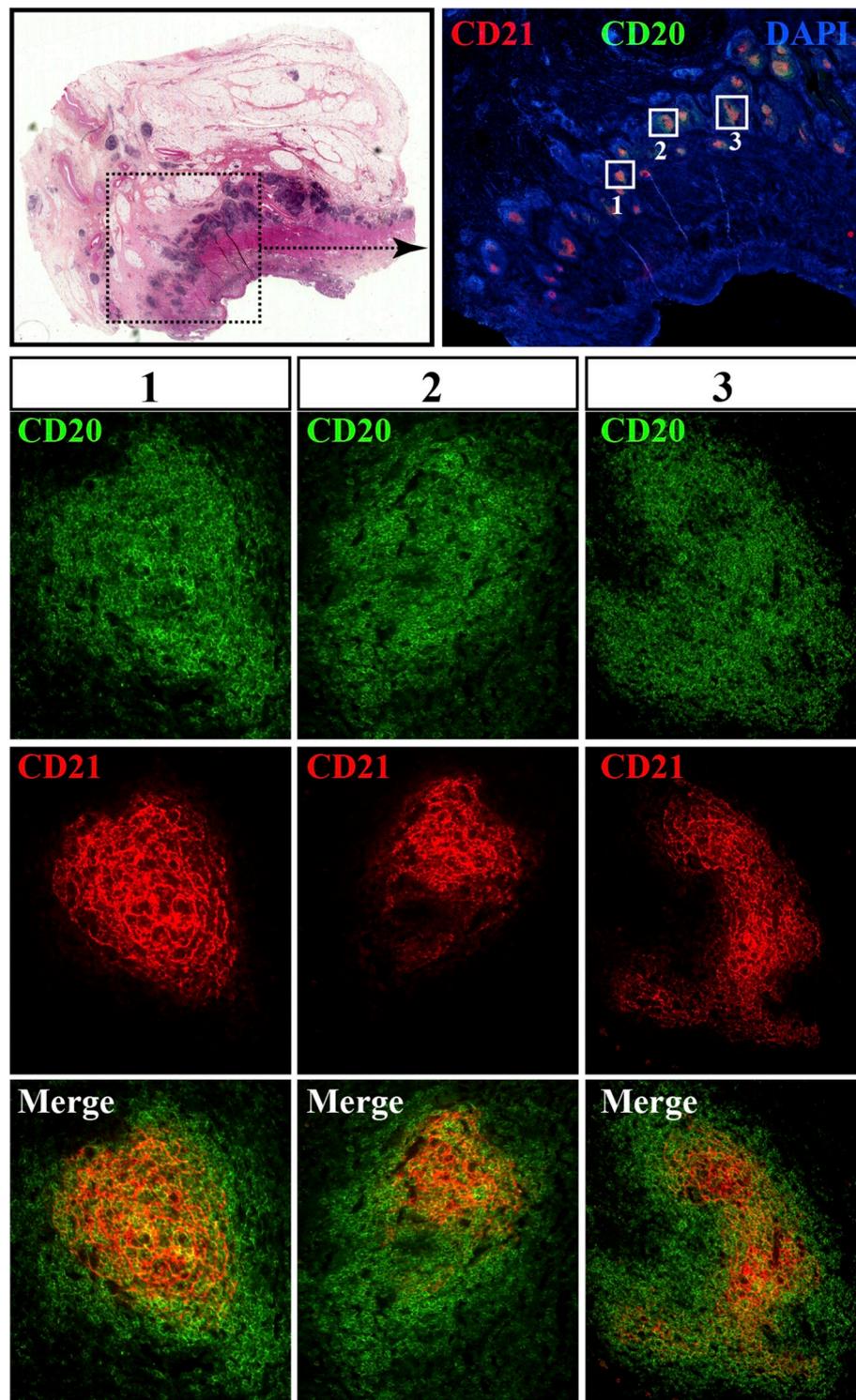
### 3.1. Fully mature TLOs develop in CD mesenteric tissues

Lymphoid nodules were detected in resected mesenteric tissues from CD patients, but not in control tissues (Supplementary Fig. 1). These nodules were mostly located under the subserous mesentery, adjacent to the deep muscle layer, sometimes they were found deeper in the mesentery, in which case they formed along the vascular peduncle. The nodule density was particularly high in the regions of the intestine where transmural damages were manifest. These nodules were composed of CD3<sup>+</sup> T cells surrounding CD20<sup>+</sup> B cell clusters, with a structural organization reminiscent of that of B cell follicles and T cell areas in SLOs (Fig. 1). Additionally, the mesenteric lymphoid structures were irrigated by CD31<sup>+</sup> blood vessels and were drained by a gp38<sup>+</sup> lymphatic network (Fig. 1). Of note, the lymphoid nodules were not surrounded by continuous gp38<sup>+</sup> lymphatic capsules, and therefore, are not SLOs. Some of the blood vessels feeding the lymphoid structures were layered with PNAd<sup>+</sup> endothelial cells displaying a cuboidal shape, demonstrating the presence of high endothelial venules, which are known to be mandatory for the recruitment of naïve and memory cells within SLOs [9].

These observations were supported by flow cytometry analysis performed on cell suspensions obtained after enzymatic digestion of the human mesenteric tissues where we found that the mesentery from CD patients was enriched in leukocytes, especially T CD3<sup>+</sup> and B CD19<sup>+</sup> lymphocytes, and neutrophils, compared with mesentery from control subjects (Fig. 2). These data strongly suggest that the lymphoid structures present in the mesentery of CD patients display key features of TLOs, and their spatial organization indicates a high degree of maturation.

### 3.2. Functional germinal centers are present in CD mesenteric tissues

To further analyze the composition of these lymphoid structures, we searched for the presence of cells known to support the function of



**Fig. 3.** CD-associated lymphoid structures contain germinal centers. At the upper-left corner, hematoxylin-eosin staining performed on human intestinal wall cross-sections from CD patients. The dashed box represents the area that is magnified in the picture at the upper-right corner, staining of CD21, CD20, and DAPI was performed on adjacent cross-sections. The boxes “1”, “2”, and “3” indicate representative mesenteric TLOs, which are magnified below.

germinal centers. Located within the B cell compartment of mesenteric TLOs, we found dense networks of follicular dendritic cells (FDCs) identified by their expression of CD21 (Fig. 3), CD23, CD35, SSTR2A and Podoplanin (Supplementary Fig. 2). In SLOs, FDCs are key cells of the germinal center reaction since they can capture and present antigens in their native forms to B cells during the selection of high affinity clones. The presence of such cells therefore suggested that mesenteric TLOs are highly mature and that they can support the local maturation,

proliferation, and selection of B lymphocytes (Fig. 3). This hypothesis was confirmed by the immunodetection of  $CD20^+AID^+$ , of  $CD20^+Bcl6^+$ , and of  $CD20^+PCNA^+$  B cells (Fig. 4). Indeed, the fact that B cells expressed AID (Activation-induced cytidine deaminase) is the demonstration that these cells are ongoing class switch recombination and somatic hypermutation of immunoglobulin genes [10]. *BCL6* gene expression is tightly regulated during mature B cell differentiation. The presence of Bcl6 protein in B cells indicate that they

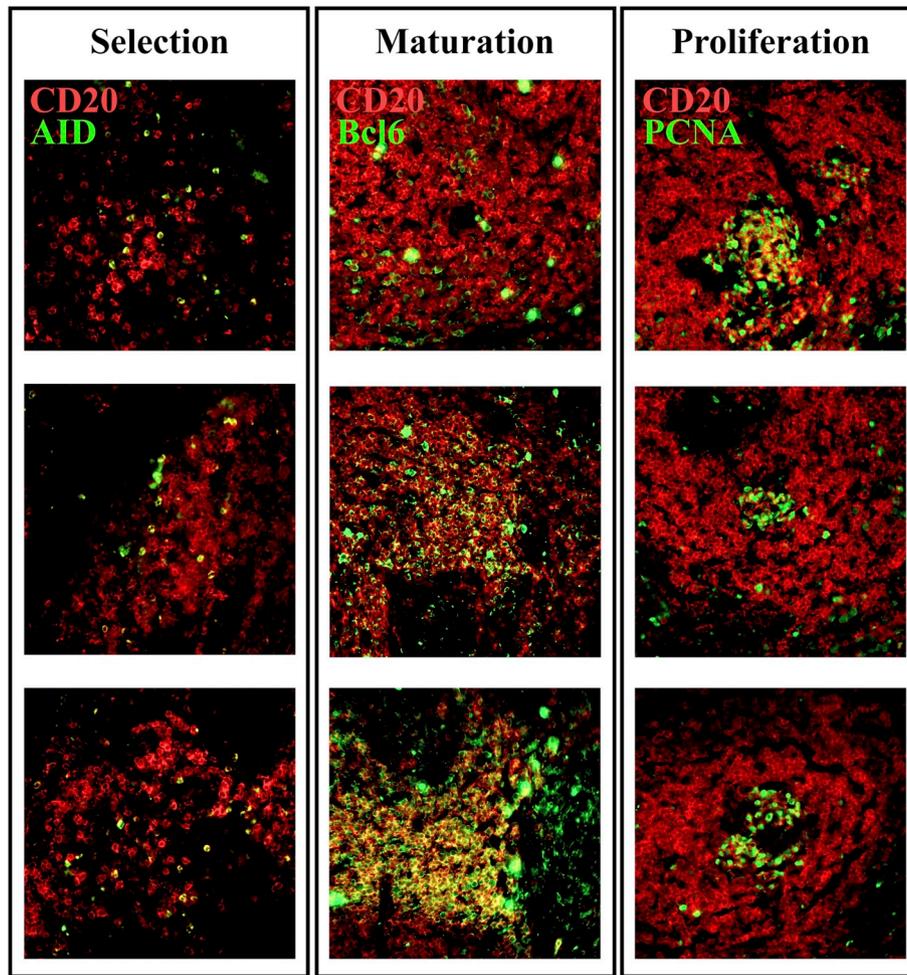


Fig. 4. CD-associated GCs present key features of selection, maturation and proliferation. Representative staining of CD20, AID, Bcl6, and PCNA performed on human intestinal wall cross-sections from CD patients at the site of supposed CD-associated mesenteric germinal centers.

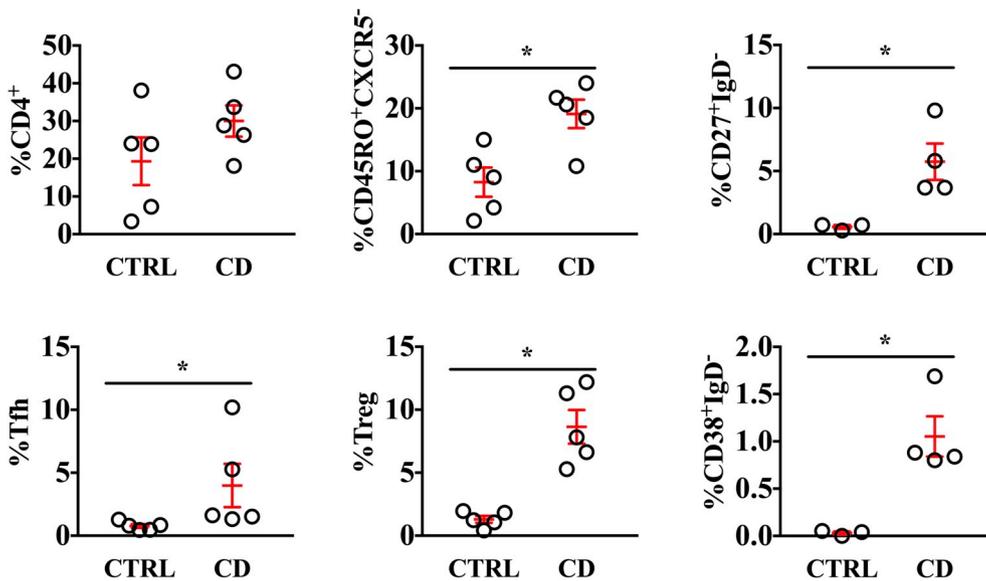
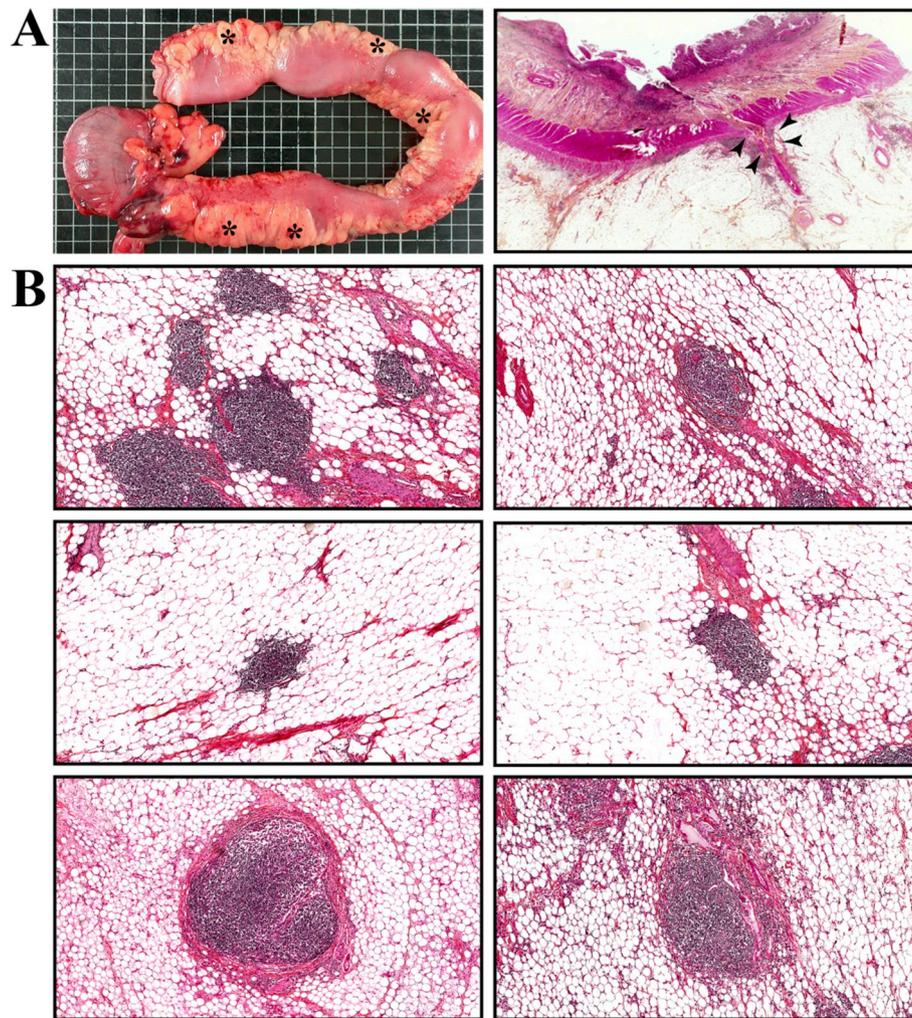


Fig. 5. Specific leukocyte populations in the mesentery of CD patients. Cytometric analysis showing the percentage of CD4<sup>+</sup>, CD127<sup>-</sup>CD25<sup>+</sup> Treg, CD127<sup>-</sup>CXCR5<sup>+</sup> Tfh, CD45RO<sup>+</sup> and CD45RO<sup>-</sup> memory and naïve cells, within T lymphocytes, and of IgD<sup>-</sup>CD27<sup>+</sup> memory B lymphocyte and CD38<sup>+</sup>IgD<sup>-</sup> plasma cells (among CD45<sup>+</sup>Live-Dead<sup>neg</sup> cells) was performed on 1 mg of digested surgical human mesenteric pieces from CD patients and from controls.

are at the germinal center stage of their differentiation (ref: [11]). The detection of CD20<sup>+</sup> B cells expressing PCNA (Proliferating cell nuclear antigen) in the TLOs is the demonstration that these B cells are undergoing proliferation. An extended flow cytometry analysis revealed an enrichment of CD19<sup>+</sup>IgD<sup>-</sup>CD27<sup>+</sup> memory B cells,

CD19<sup>+</sup>IgD<sup>-</sup>CD38<sup>+</sup> plasma cells, as well as CD4<sup>+</sup>CD45RO<sup>+</sup> memory and CD3<sup>+</sup>CD4<sup>+</sup>CD127<sup>+</sup>CXCR5<sup>+</sup> follicular helper T cells in the mesentery of CD patients compared with the mesentery of the controls (Fig. 5; please see Supplementary Fig. 3 for the full gating strategy), thereby further showing that TLOs sustain local germinal center



**Fig. 6.** Mesenteric TLOs are systematically embedded in hypertrophic adipose tissue. (A) On the left side, macroscopic picture of an intestinal segment after resection in a patient with CD, showing hypertrophic adipose tissue (asterisks). On the right side, hematoxylin-eosin staining performed on human intestinal wall cross-sections from CD patients, showing perforation of the intestinal wall (arrowheads). (B) Hematoxylin-eosin staining performed on human intestinal wall cross-sections from CD patients showing the ubiquitous embedment of mesenteric TLOs within the adipose tissue.

reactions.  $CD4^+CD127^-CD25^+$  T regulatory cells were also detected, indicating that immune responses taking place in CD's mesenteric TLOs might be, at least partially, controlled.

### 3.3. The mesenteric adipose tissue as the organizer of TLOs

As exemplified in Fig. 6A, the mesenteric adipose tissue is dramatically hypertrophied in CD patients. Importantly, TLOs were located within this tissue, in close proximity with the muscular layer of the intestinal wall, with a high prevalence where transmuscular damages were observed (Fig. 6B). They were also observed around the vascular peduncles spanning the mesentery. The almost systematic embedding of TLOs into the adipose tissue suggested that chemokines able to attract T and B cells were produced within this tissue. Indeed, the expression of CCL19 ( $P < 0.05$ ), CCL20 (trend), CCL21 (trend), CXCL13 (trend), and CXCL16 ( $P < 0.05$ ) chemokines in mesenteric tissues adipocytes was higher in tissue from CD patients as compared to control intestinal samples (Fig. 7A). In functional chemotaxis assays, we found that soluble mediators released by CD mesenteric pieces (conditioned-media) could specifically trigger the recruitment of human  $CD19^+$  B cells, compared with that of conditioned-media prepared from control tissues (Fig. 7B).

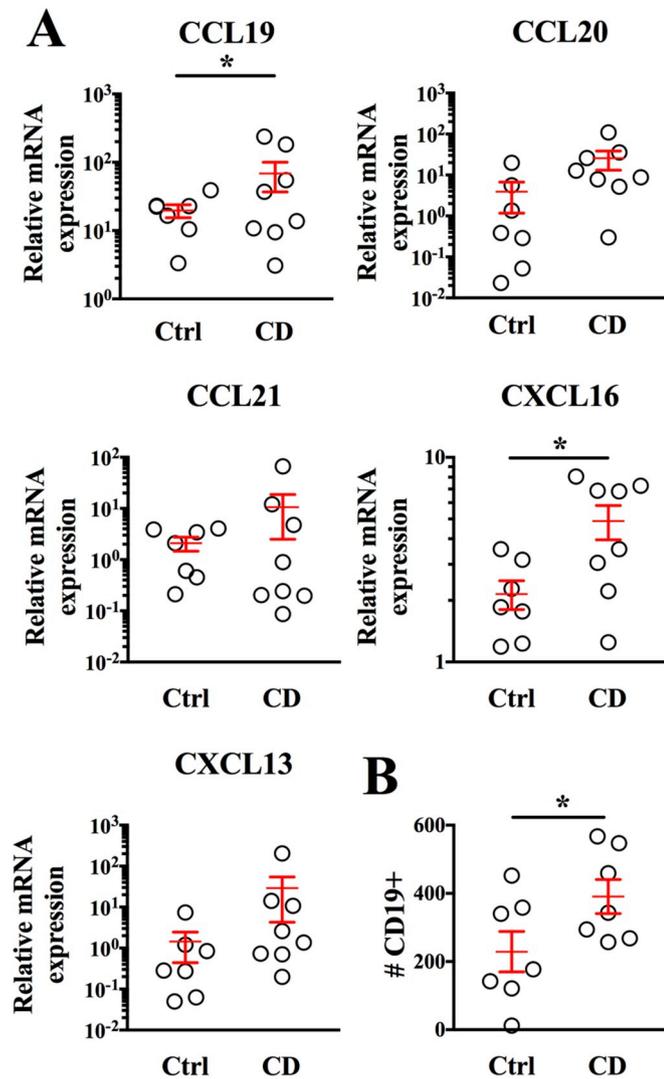
Interestingly, the chemokine-producing cells were distributed according to specific patterns, as detected by immunohistochemistry

(Supplementary Fig. 4). CXCL16 and CCL20 chemokines were preferentially found in the T lymphocyte zone, while CCL21 and CXCL13 chemokines were restricted to the B cell area. CCL19 chemokine was found to be equally distributed in the two zones. These observations suggest that CXCL16, CCL20, CCL21, and CXCL13 play a central role in the establishment/maintenance of the B cell/T cell compartmentalization.

Strikingly, we also found that adipose cells proximal to the TLOs were strongly positive for the CCL20 and CXCL13 chemokine (Fig. 8A), which also colocalized with intracellular actin staining (Supplementary Fig. 5), thereby indicating that these stromal cells participate to the chemoattraction of B and T cells. This could explain why the mesenteric adipose tissue in CD is favorable for the development of TLOs. Additionally, this suggested that adipose cells could participate in the process of mesenteric TLO formation.

### 3.4. $TNF-\alpha$ and LPS trigger the recruitment of immune cells by mesenteric adipocytes

As mentioned above, the mesenteric territory wherein TLOs develop is anatomically close to deep ulcerations and fistulae in the inflamed intestine of CD patients. Therefore, the mesenteric tissue of CD patients is in contact both with inflammatory molecules such as  $TNF-\alpha$  (which is increased in the serum of patients [12]), and with bacterial components



**Fig. 7.** The mesentery in CD produces specific chemokines capable of triggering the recruitment of leukocytes. (A) The expression level of chemokines was analyzed in whole human mesenteric surgical pieces from CD patients and from controls by RT-qPCR on extracted RNA. Data were analyzed using the  $2^{-\Delta\Delta C_t}$  Pfaffl formula [21], in which  $C_t$  values from control patients were compared with those from CD patients and normalized to the  $C_t$  values of ACTB. (B) Chemotaxis assay on conditioned-media prepared from human mesenteric surgical pieces of CD patients and from controls. Plotted is the number of CD19<sup>+</sup> B cells that have migrated toward the wells containing the conditioned-media. \*p < 0.05.

such as LPS. Of note, during lymphoid (neo)genesis, TNF- $\alpha$  confers stromal cells with a LTo potential [13,14].

We therefore tested whether TNF- $\alpha$  and/or LPS could trigger the expression of chemokines in adipocytes. The expression of CCL19, CCL21, CXCL13, CCL20 and CXCL16 was analyzed by RT-qPCR in murine 3T3-L1- adipocytes, stimulated or not by TNF- $\alpha$  or LPS. We found that TNF- $\alpha$  strongly induced the expression of all but one (CCL21) chemokine (Fig. 8B). Besides, LPS induced a strong expression of CCL21 and CCL19. These results were corroborated by the measurement of the chemokines in the cell culture supernatants, except for CCL21 for which we failed to detect increased protein levels, regardless of the culture condition (Fig. 8C). The combination of LPS and TNF- $\alpha$  is therefore expected to be optimal to confer adipocytes with a full LTo cell potential.

Finally, we found that the supernatant of 3T3-L1 cells stimulated with TNF- $\alpha$  and LPS induced the recruitment of several subpopulations

of T and B cells, while the supernatant of unstimulated cells did not have such an effect (Fig. 8D).

#### 4. Discussion

Herein, we report that the mesenteric tissue of CD patients is enriched in memory lymphocytes and plasma cells, and hosts TLOs. CD mesenteric TLOs displayed compartmentalized B and T cell areas that were irrigated by blood vessels layered by PNA<sup>+</sup> endothelial cells displaying a cuboidal shape. These vessels are probably high endothelial venules. PNA<sup>+</sup> high endothelial venules are specialized for the recruitment of CD62L<sup>+</sup> lymphocytes in SLOs (besides spleen) and in the thymus. They likely play the same role in TLOs for the regulation of the entry of naïve and memory lymphocytes. Mesenteric TLOs in CD patients also displayed a network of CD21<sup>+</sup> FDCs, indicating ongoing local B cell responses. Indeed, FDCs have the capacity to capture and to coat their surface with antigens on which B cells can test their B cell receptors during the germinal center reaction through which the selection of high affinity B cell clones occurs [15].

We next tried to decipher why TLOs preferentially developed in the creeping fat of the mesentery [7], a tissue that is a deeply remodeled with a massive adipose tissue expansion in CD patients. The extent of this creeping fat (or “sclerolipomatosis”) is such that it guides the surgeon in delineating the intestinal segment that requires resection. Our data suggest that the localization of the TLOs in this tissue is due to the local high concentration of CCL19, CCL20, CCL21, CXCL13, and CXCL16 chemokines, likely produced by the adipocytes in the vicinity of TLOs, as well as by the cells comprised within the TLOs themselves, further sustaining their implantation.

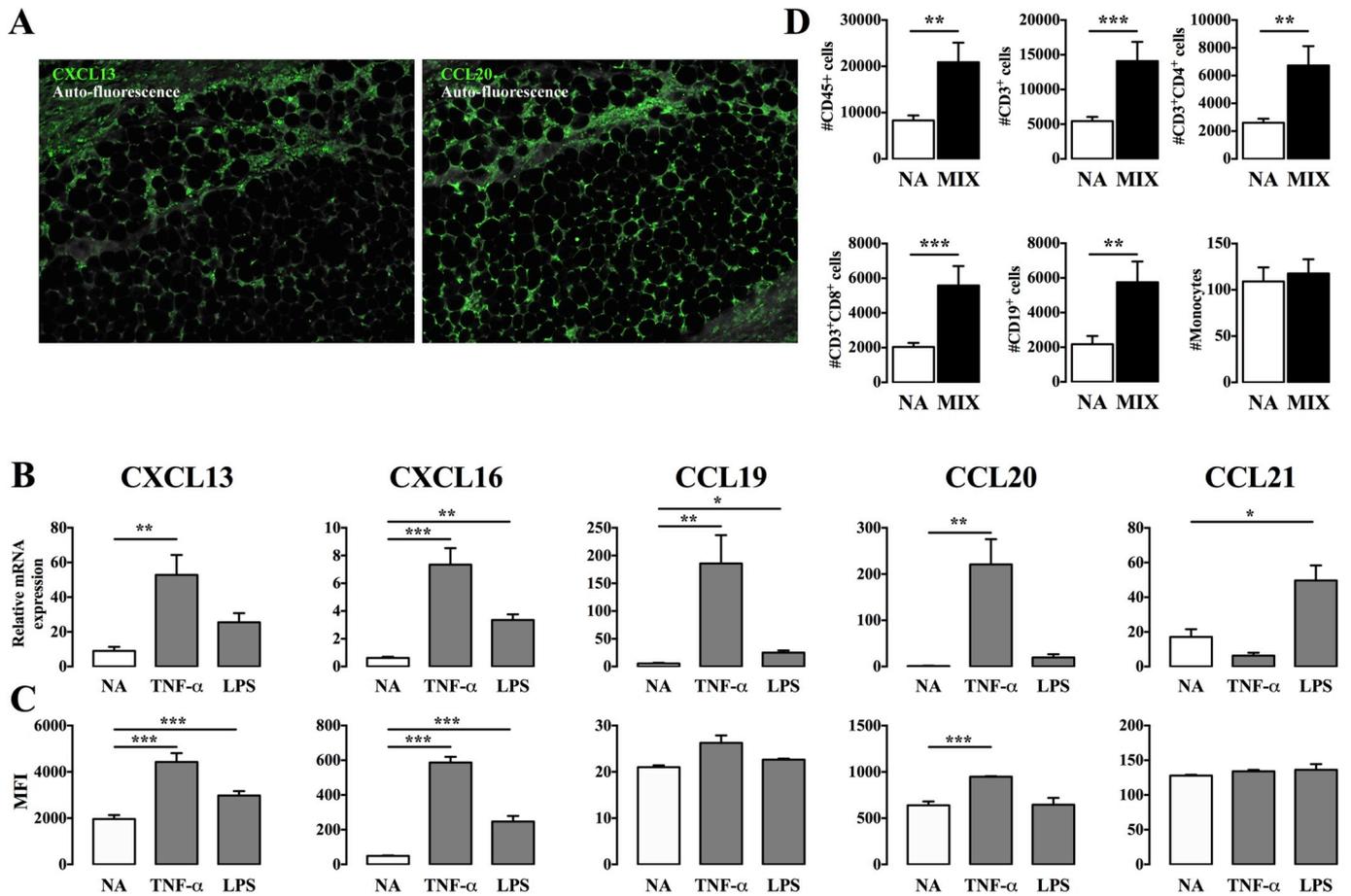
Thus, similar to what occurs in chronically inflamed arteries [13,14,16,17], where the most abundant stromal cells, the vascular smooth muscle cell, adopt the function of TLO organizer cells, in the sclerolipomatous tissue of CD patients where TLOs are embedded, the most abundant stromal cell of the mesenteric fat, adipocytes appear able to orchestrate the development of fully mature TLOs. The highly plastic adipose cells were already known to be able to produce vast amounts of a large panel of cytokines [18]. It is not surprising that they can also produce chemokines.

While our vitro experiments demonstrate that adipocytes can produce these chemokines, we recognize that the immunodetection of chemokines in adipocytes from human CD mesenteric tissue is not a definitive demonstration that they do so since these cells could have taken up these chemokines. Further investigations are required in order to rule out whether different stromal cells, other than adipocytes, could also be endowed with lymphoid tissue-organizer capacities in CD mesenteric tissue.

Stimulation with TNF- $\alpha$  alone however does not seem to be sufficient to trigger the organizer cell function of adipocytes, the addition of LPS was necessary in order to produce the full panel of chemokines required to recruit the different cell populations that form TLOs. This finding may explain why CD-associated TLO develop close to the sites of lesions of the intestinal barrier, where bacterial-derived inflammatory stimuli can act in a synergistic manner with the TNF released by the damaged tissue to trigger the production of homeostatic chemokines by the local adipocytes.

CD is not the sole intestinal pathological condition associated with an alteration of the intestinal barrier. Indeed, the translocation of bacterial components can be observed in other enteritis such as radiation enteritis, tuberculosis enteritis and ischemic enteritis. Thus, the “mesenteric inflammatory environment” is not specific to CD. At variance, the sclerolipomatosis is pathognomonic of CD. The presence of the wrapping fat subjected in an inflammatory environment triggered by an altered epithelial barrier would therefore constitute the key combination for the development of TLOs specifically in the mesentery of CD patients.

Concerning the impact of mesenteric TLOs, whether they are solely



**Fig. 8.** Adipocytes play a central role in mesenteric lymphoid neogenesis. (A) Representative staining of DAPI, CD20, and chemokines performed on human intestinal wall cross-sections from CD patients showing the expression of chemokines in mesenteric adipocytes localized at the vicinity of TLOs. (B, C, D) Murine 3T3-L1 cells were stimulated for 15 h with rTNF-α and LPS. (B) The expression levels of chemokines were determined by RT-qPCR on extracted RNA. Data were analyzed using the  $2^{-\Delta\Delta C_t}$  Pfaffl formula [21], in which  $C_t$  values from stimulated adipocytes were compared with those from unstimulated cells and normalized to the  $C_t$  values of HPRT. (C) The concentrations of chemokines were assessed using cytometric beads on the supernatant of stimulated and unstimulated cells. (D) Chemotaxis assay of the supernatant of 3T3-L1 cells stimulated for 15 h with rTNF-α + LPS. Plotted is the relative number of CD45<sup>+</sup> hematopoietic cells, CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> helper and cytotoxic T lymphocytes, CD19<sup>+</sup> B lymphocytes, and monocytes that have migrated toward the 3T3-L1 supernatant-containing wells. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001.

protective structures restraining the bacterial entry across the damaged intestinal epithelium or instead play a role in the perpetuation of the intestinal damage will require further studies. It is worth mentioning that immune responses mounted in TLOs develop in the inflamed tissue and outside of controlled lymphoid organs. As a consequence, stochastic and potentially harmful immune responses emerge from TLOs [19]. We however found Treg cells the mesenteric of CD patients indicating that the local immune responses may be regulated to some extent. This regulation might be insufficient and TLOs could play deleterious role in CD since we found that the mesenteric lymphoid structures were associated with a more severe phenotype of CD (a study that is currently submitted).

As a perspective to our findings, monitoring the formation and/or the expansion of TLOs in longitudinal studies (by cellular/molecular imaging of B cells or other TLO-specific molecules) should be considered for clinical staging of CD. Finally, targeting the formation of TLOs and/or the function of the immune effectors generated locally appears as a new promising strategy to impact on the pathologic process of CD. An additional way to impact on mesenteric TLOs is the resection of the mesentery support tissue. Indeed, it was recently shown that removing the mesentery during ileocolic resection in CD patients reduced post-operative recurrence [20]. This lends support to our idea that the mesentery and the immune response that it hosts play a crucial role in the pathophysiology of CD.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jaut.2019.05.009>.

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