

Specialized macrophages link gut lymphoid follicles to Crohn's disease granulomas

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Abstract

Granulomas are a defining feature of Crohn's disease associated with a more severe disease course, yet their developmental origins and cellular organization remain poorly understood. Here, we integrate single-cell and spatial transcriptomics of human intestinal tissues with *in situ* validation and functional macrophage assays to investigate granuloma formation. Crohn's disease granulomas share cellular, transcriptional and spatial features with isolated lymphoid follicles, suggesting that chronic inflammation may repurpose pre-existing lymphoid structures. We identify a previously uncharacterized population of PLA2G2D-expressing macrophages enriched in isolated lymphoid follicles and at granuloma peripheries. Lymphotoxin- α 1 β 2, LIGHT and TNF induce this macrophage programme *in vitro*, while TNF and low-dose lipopolysaccharide promote CXCL9 production together with PLA2G2D expression, potentially supporting effector T-cell recruitment in the granuloma environment. Conversely, SPP1-expressing macrophages preferentially occupy granuloma cores, are induced by IL-1 β or hypoxia, and interact with inflammatory fibroblasts through profibrotic signalling networks. These findings define spatially and functionally distinct macrophage niches within Crohn's disease granulomas and support a model in which inflammatory signals transform homeostatic lymphoid macrophage programmes to promote granuloma organization and fibrotic remodelling.

Summary

Granulomas are a defining feature of Crohn's disease associated with a more severe disease course, yet their developmental origins and cellular organization remain poorly understood. Here, we integrate single-cell and spatial transcriptomics of human intestinal tissues with validation and functional macrophage assays to investigate granuloma formation. Crohn's disease granulomas share cellular, transcriptional and spatial features with isolated lymphoid follicles, suggesting that chronic inflammation may repurpose pre-existing lymphoid structures. We identify a previously uncharacterized population of PLA2G2D-expressing macrophages enriched in isolated lymphoid follicles and at granuloma peripheries. Lymphotoxin- α 1 β 2, LIGHT and TNF induce this macrophage programme , while TNF and low-dose lipopolysaccharide promote CXCL9 production together with PLA2G2D expression, potentially supporting effector T-cell recruitment in the granuloma environment. Conversely, SPP1-expressing macrophages preferentially occupy granuloma cores, are induced by IL-1 β or hypoxia, and interact with inflammatory fibroblasts through profibrotic signalling networks. These findings define spatially and functionally distinct macrophage niches within Crohn's disease granulomas and support a model in which inflammatory signals transform homeostatic lymphoid macrophage programmes to promote granuloma organization and fibrotic remodelling.

INTRODUCTION

As the body's largest immune organ, the intestine exhibits a remarkable diversity of immune cells that must be tightly controlled. Central to this regulation are isolated lymphoid follicles (ILFs), which serve as adaptive immune-inductive sites promoting tolerance towards luminal antigens^{1, 2}. This equilibrium is

severely compromised in Inflammatory Bowel Disease (IBD), specifically in ulcerative colitis (UC) and Crohn's disease (CD). UC and CD are chronic intestinal disorders that arise from complex interactions between genetic, environmental, and immunoregulatory factors, although the specific etiology remains unknown^{3, 4}. Persistent immune activation is associated with the development of ectopic tertiary lymphoid structures (TLS) which are organized immune aggregates that develop in non-lymphoid tissues under pathological conditions⁵. In contrast to TLS that are a shared feature of UC and CD, granulomas (GA) are only seen in 15–50% of CD patients^{6–9} and they are associated with a more aggressive disease phenotype; i.e.: increased risk of repeat surgery¹⁰. GA were in fact reported in the original description of the disease by Crohn et al¹¹ as a hallmark histologic feature. These aggregates of immune cells, predominantly macrophages, surrounded by lymphocytes, arise to isolate persistent antigens¹². Although evolutionarily designed for protection against microbes, GA can emerge without a known initial trigger to drive pathological fibrosis and organ damage in non-infectious diseases¹³. Beyond their traditional role in containment, recent studies in sarcoidosis have reframed GA as aberrant lymphoid organs¹³. Similarly, tuberculosis models describe a functional association between protective GA and the formation of B-cell-rich lymphoid structures, highlighting the structural and functional interconnection between these two. Despite these advances, the pathogenesis and role in disease progression of GA in non-infectious diseases remains largely unknown.

Here we investigate the changes that occur in lymphoid cell-rich structures within the intestine of IBD patients, and their common and unique features compared to CD-associated GA. By combining scRNA-seq derived annotations with spatial transcriptomics of human intestine we reveal the main cellular identities, particularly macrophage subsets, present in ILFs and GA. Based on analysis of cellular composition and cell-to-cell interactions we uncover potential signals driving or maintaining these structures in inflamed tissues. Both their transcriptional signatures and tissue locations strongly suggest that CD-associated GAs may arise from immune-rich TLSs.

RESULTS

Neighborhood analysis of healthy and inflamed colonic sections identifies lymphoid aggregates and granuloma as distinct structures

To unravel changes in the architecture of the colon of patients suffering from IBD, we took advantage of a spatial transcriptomics dataset we previously generated¹⁴ (Figure 1A). Collectively, this dataset contained 583,974 cells which we re-annotated here using a larger in-house scRNA-seq dataset as reference including 92 colonic samples from 70 donors (Table S1). The annotated scRNA-seq object contained 5 cellular clusters comprising epithelial, stromal, myeloid, T cells and B/Plasma cells. Further clustering revealed 71 total cell types or states across all 5 compartments (Figure S1). Next, we transferred cell type labels onto the spatial data which resulted in 48 different cell identities being localized in tissues (Table S2).

To gain insight into tissue organization, we applied neighborhood analysis following a previously described methodology¹⁵. In brief, we identified a total of 21 statistically recurrent cellular neighborhoods across all samples (Figure 1B). Only 10 of these neighborhoods were present in healthy colon, whereas IBD samples exhibited all 21 at varying proportions (Figure 1C). As seen in mice¹⁵, neighborhood composition was highly conserved across healthy colon but was altered in IBD with high inter-sample variation, highlighting disease heterogeneity and reflecting marked disruption of tissue organization in both UC and CD samples (Figure 1B and 1C).

Based on their cellular composition, we could differentiate 3 neighborhoods highly enriched for T cells, which also contained abundant B cells, fibroblastic reticular cells (FRCs), dendritic cells (DCs), plasmacytoid DCs (pDCs), and macrophages in different states (Figure 1D). Given their structure and anatomical localization, we labeled 2 of these neighborhoods as ILFs and the less abundant surface ILFs (SurILF), mainly formed by B cells and plasmablasts adjacent to ILFs. As expected, ILFs and SurILFs were found in both healthy and inflamed colon (Figure 1C). In contrast, the third T cell-rich neighborhood was exclusively found in diseased samples, particularly in one of the 3 CD samples (CD2, Figure 1B). Besides T cells, these structures comprised abundant myeloid cells including DCs and several types of macrophages which were preferentially positioned at the center of the structures and surrounded by T cells with inflammation-associated fibroblasts (IAFs) interspersed throughout (Figure 1E and Figure 2A). Based on this and confirmation by two expert pathologists (MC and AC), we annotated these structures as GA.

Comparison between healthy and IBD isolated lymphoid follicles reveals disease-associated changes in cellular composition and gene expression

While ILFs are present in healthy colon, they tend to proliferate in number and increase in size in the context of chronic inflammation^{2, 5}. The mechanisms driving the reorganization or *de novo* generation of these complexes also known as TLS are poorly understood. To determine the changes that accompany this remodeling, we compared ILFs (identified by neighborhood analysis) in healthy and inflamed (UC or CD) colon by measuring their cellular composition and differentially expressed genes (DEGs). In IBD_ILFs most significantly increased cell types included IgG plasma cells (FDR=9.29 x10⁻⁷), plasmablasts (FDR=3.20 x10⁻⁶), and IAFs (FDR=3.39 x10⁻⁴), while the proportions of T lymphocytes, despite a trend towards decreased naïve T cells, were not significantly changed compared to ILFs in healthy colon (Figure 2B) (Table S3). In agreement with these, DEG analysis showed the significant increase in IBD_ILFs of *IGHG1* and *IGHG2* encoding for IgG1 and IgG2 heavy chains as well as the IAF marker *CHI3L1* (Figure 2C and 2D). In addition, in IBD, ILFs expressed significantly less *CCL21*, but not *CCL19* (Figure 2C and 2D), both CCR7 ligands mediating naïve lymphocyte recruitment and/or movement within ILFs.

As expected, changes in cellular composition and gene expression had an impact on the number and type of interactions within ILFs in HC and IBD. The overall number of interactions predicted based on receptor-ligand expression and spatial localization¹⁶ was significantly decreased in IBD compared to healthy ILFs, including those involving CCL19-expressing FRCs (Figure S2A-C; Table S4). IBD_ILFs

showed *de novo* interactions such as those mediated by IAFs suggesting a previously undescribed role for this fibroblast subset in TLSs (Figure S2D).

Crohn's disease granuloma and isolated lymphoid follicles exhibit shared and unique cellular patterns

As seen in IBD_ILFs, the relative proportion of plasmablasts, IgG plasma cells, IAFs and several macrophage populations was further increased in GA compared to healthy ILFs (Figure 2B), suggesting common drivers in both structures in the context of disease. Also, similarly to ILFs, the presence of FRCs (CosMx markers *CCL19*, *CCL21*, *PTGDS*) and lymphatic endothelial cells (CosMx markers *CCL21*, *ADGRG3*), although less prevalent, was also observed in GA (Figure 2B, Table S2). A novel observation of our analysis is the presence of a subset of macrophages characterized by expression of secreted phospholipase A2 group IID (*PLA2G2D*) and found predominantly in ILF, both in healthy and IBD colon, and GA (Figure 1D and Figure 2A-B). *PLA2G2D*⁺ macrophages were distinct from previously described resident M0 and FOLR2⁺ (folate receptor 2) or inflammatory (M1) macrophages (Table S2).

Despite these similarities with ILFs, GA presented unique features, including a significant reduction in the abundance of naïve T cells, Treg, ILC3 and B cells, and an overall increase in the proportion of macrophages compared to ILFs (Figure 2B). Macrophage subsets found in GA included FOLR2⁺, *PLA2G2D*⁺ as well as *SPP1*⁺ macrophages (Figure 3A) which expressed both inflammatory and profibrotic markers (CosMx markers *SPP1*, *MT1X*, *S100A8*, *MT2A*, *S100A9*) (Table S2). Most of the interactions predicted to take place within GA involved macrophages as either senders or receivers emphasizing their important role in GA (Figure S2E). Finally, DEG analysis showed the three most significantly upregulated genes in GA compared to ILFs to be *CHI3L1* (expressed by IAFs), *S100A8* (*SPP1*⁺ macrophages) and *IGHG2* (IgG Plasma cells) (Figure 2E), in agreement with the increase in the proportion of all these cellular subsets (Figure 2B). A significant reduction in expression of the naïve B cell marker gene (*IGHD*) as well as *IGFBP3* (expressed by endothelial cells, fibroblasts and FRCs) was also evidenced in GA compared to ILFs (Figure 2E).

We conclude that TLS (or ILFs) and GA share some features, including the enrichment of lymphoid tissue-associated *PLA2G2D*⁺ macrophages, T cells and FRCs seeding both structures, while they differ in the proportion of *SPP1*⁺ macrophages expressing pro-inflammatory/anti-microbial and pro-fibrotic markers.

***PLA2G2D* expression identifies a macrophage subset enriched in both isolated lymphoid follicles and Crohn's disease-granuloma that is distinct from *SPP1*⁺ macrophages**

Spatial analysis resolved the neighborhood-dependency of certain macrophage populations. Indeed, *PLA2G2D*⁺ macrophages were abundant within lymphoid-rich structures (including both ILFs and GA), while *SPP1*⁺ macrophages were found primarily in GA and inflamed areas of the mucosa or submucosa (InfStroma neighborhood) (Figure 3A). Cell-cell proximity enrichment analysis, independent of neighborhood restrictions, confirmed that *PLA2G2D*⁺ macrophages co-localized with B cells, DCs and T

cells supporting their enrichment in inductive lymphoid tissues (Figure 3B). In contrast, *SPP1*⁺ macrophages were found in proximity to other macrophage subsets, including *M0*, *M1*, *FOLR2*⁺ and *PLA2G2D*⁺, and to DCs (Figure 3B). To further investigate the association of *PLA2G2D*⁺ macrophages with lymphoid-rich tissues we analyzed a published human tonsil atlas¹⁷ and found high expression of *PLA2G2D*, together with *MMP9* and *PTGDS* (both markers of *PLA2G2D*⁺ macrophages in the CosMx dataset), by lymph node macrophage populations (Figure S3A) in agreement as well with our intestinal scRNA-seq dataset (Figure S3B). Despite its association with lymphoid structures, *PLA2G2D*⁺ macrophages did not express markers (*SIGLEC1*/*CD169* or *CD36*) previously identified as expressed by macrophages in lymph nodes¹⁸ or human tonsils¹⁹, respectively (Figure S3C).

We further validated the site-specific expression of *PLA2G2D* by ISH in samples from an independent group of patients (Table S1). This analysis confirmed the localized expression of *PLA2G2D* mRNA by *CD68*⁺ macrophages in ILFs but not in the lamina propria of non-IBD colon (Figure 3C). *PLA2G2D* was also highly expressed by macrophages in IBD_ILFs (Figure 3D-3F) and GA (Figure 3F) but rarely found in the inflamed lamina propria.

Also, in agreement with data from spatial transcriptomics, *SPP1* was detected as described previously²⁰ at the core of GA but not in HC (Figure 3C) or IBD_ILFs (Figure 3D-F). Besides GA, *SPP1* was expressed by cells in ulcerated mucosa, where *PLA2G2D* was absent or scarcely expressed (Figure S4A). Finally, in agreement with scRNA-seq data (Figure S4B), *SPP1* was also detected in *CD31*⁺ vascular endothelial cells in lamina propria (Figure S4C) and ILFs (Figure S4D), particularly in UC (Figure 3E, lower panel).

In summary, we show that *PLA2G2D*⁺ and *SPP1*⁺ macrophages exhibit preferential independent locations with the first displaying a strong association with ILF and TLS in intestinal tissue. ILFs, instead, were devoid of *SPP1*⁺ macrophages even in the context of active IBD. Intriguingly, GA hosted both *PLA2G2D*⁺ and *SPP1*⁺ macrophages, suggesting a unique environment that favored the co-localization of these populations in GA.

Unique signals induce the polarization of *PLA2G2D*⁺ and *SPP1*⁺ macrophages

To further understand the conditions that are associated with the presence of each macrophage subset, we leveraged bulk RNA-seq data from a cohort of healthy and IBD biopsies (GSE235236) and determined which genes correlated with either *PLA2G2D* or *SPP1* expression. Both genes were significantly upregulated in active IBD colonic samples compared to healthy colon (Figure S5A). Correlation analysis identified independent signatures to significantly correlate with each of these macrophage subset-specific markers (Table S5). Top 10 correlated genes are shown for CD (Figure 4A) and UC (Figure S5B). Pathway analysis of each of these signatures showed genes positively correlated with *PLA2G2D* expression to participate in T-cell signaling pathways (Figure 4B), while signals induced by cytokines including IL-4, IL-1, TNF and Oncostatin M were found to correlate with *SPP1* expression in bulk RNA from IBD biopsies (Figure 4C). Among the genes highly correlated with *PLA2G2D*, we found those

encoding for the lymphotoxin subunits (*LTA* and *LTB*) (Figure 4D), which are all lymphoid tissue inducing signals, further supporting the association of this macrophage subset with organized lymphoid tissues and suggesting these signals could contribute to the induction of the *PLA2G2D*⁺ macrophage-phenotype²¹⁻²³. To test this hypothesis, we cultured monocyte-derived macrophages with *LT* $\alpha_1\beta_2$, *LIGHT* or *TNF* (Figure 4E). All three cytokines significantly increased expression of *PLA2G2D* and *MMP9*, but not *SPP1* (Figure 4E). Instead, expression of *SPP1* on macrophages was induced in response to *IL-1* β or hypoxia, in agreement with previous studies²⁰⁻²². Interestingly, *IL-1* β , which increased *SPP1* mRNA on macrophages, significantly downregulated *PLA2G2D* expression. Thus, as suggested by their distinct localization, the *PLA2G2D* and *SPP1* programs appear to be mutually exclusive and driven in response to independent signals.

***PLA2G2D*⁺ and *SPP1*⁺ macrophages display different cell-to-cell interactions within granuloma**

While signals driving each phenotype of macrophages were distinct, we were intrigued by the observation that *PLA2G2D*⁺ and *SPP1*⁺ macrophages could co-exist in GA, but not in ILFs where *PLA2G2D*⁺ macrophages were highly enriched as well. We then investigated the receptor-ligand interactions involving these macrophage subsets in ILF and GA. Remarkably, we did not detect any significant interaction (using a cutoff above 60% likelihood) involving macrophages in non-IBD ILFs (all interactions can be seen in Figure S2), while only a few were found in ILFs from one of the CD patients analyzed, which also showed GA (Figure 4F). The low proportion of interactions involving macrophages likely reflects their relative scarcity in these structures compared to more abundant subsets, including B cells, T cells, and FRCs. In contrast to ILFs, within GA, *FOLR2*⁺, *PLA2G2D*⁺ and *SPP1*⁺ macrophages engaged in significant interactions (74.2% of total interactions, >60% likelihood) (Figure 4F, Table S4).

PLA2G2D⁺ macrophages interacted exclusively with B cells (which are the main *LT* $\alpha_1\beta_2$ producer, Figure S5C) in ILF (Figure 4F). Compared to ILFs, *PLA2G2D*⁺ macrophages in GA showed significant interactions with T cells, including cytotoxic cells, and with IAFs. Remarkably more than 80% of the interactions established by *SPP1*⁺ macrophages involved IAFs, while no significant interaction was detected between *SPP1*⁺ macrophages and T or B cells.

Overall, our analysis combining transcriptional signatures with spatial location of cells suggests that *PLA2G2D*⁺ macrophages in GA gain interaction with T cells, which supports a role for this cell subset in attracting and/or activating T cells to the GA. Indeed, comparison with healthy ILFs, shows a significant decrease in Tnaïve and Tregs in GA (Figure 2B). In contrast, *SPP1*⁺ macrophages display no apparent contact with T cells but are instead strongly associated with IAFs showing significant interactions through profibrotic signals (*TGFB1-CAV1*, *COL6A3/COL6A2/COL6A-ITGA1* or *COL1A1/FN1/VIM/MMP1/MMP9-CD44*) (Figure S6).

TNF and LPS drive the *PLA2G2D*⁺ *CXCL9*⁺ macrophage phenotype characteristic of GA

Based on these observations, we proposed that under certain conditions $PLA2G2D^+$, but not $SPP1^+$ macrophages, can contribute to effector T cell recruitment, a key feature of GA development. We then investigated the chemokine expression patterns of $PLA2G2D^+$ macrophages based on spatial transcriptomics. *CXCL9*, a chemoattractant for activated CXCR3-expressing T cells, is one of their top markers (p.adj= 1E-300) in our dataset, with *CXCL10* also expressed but less significantly (p.adj= 8,87E-18). In situ hybridization showed expression of *CXCL9* by $CD68^+$ cells in inflamed mucosa (i.e. ulcers) in CD (Figure S4A), and in the LP of UC (Figure S4E), with patchy (in ILFs) and rare expression (in lamina propria) in healthy gut (Figure S4F). Importantly, $PLA2G2D^+$ macrophages in GA, but not those in adjacent ILFs expressed abundant *CXCL9* (Figure 5A). This was confirmed using single-molecule fluorescence in situ hybridization (smFISH) in a sample from a granulomatous CD patient (Figure 5B). Detection of *CD3E* shows T cells to co-localize with *CXCL9* macrophages in the outer ring of GA (Figure 5B). Additional staining in two independent CD patients shows *CXCL9* macrophages in GA in close contact to CD3 T cells (Figure 5C).

We next measured expression of *CXCL9* by *in vitro* activated macrophages under conditions shown above to induce *PLA2G2D* or *SPP1*. $LT\alpha_1\beta_2$ and, to a higher degree, TNF significantly increased transcription of *CXCL9*, while hypoxia negatively regulated *CXCL9* (Figure 5D). Thus, while $LT\alpha_1\beta_2$ and LIGHT drive *PLA2G2D* expression, TNF induces the highest degree of expression of both *PLA2G2D* as well as *CXCL9* on *in vitro* differentiated macrophages. Remarkably, TNF drove the significant increase of *S100A8* and *MT1X* expression as well, suggesting this cytokine could induce, in addition to *PLA2G2D* and *CXCL9*, a potent anti-microbial phenotype on $PLA2G2D^+$ macrophages (Figure 5D). Finally, we measured protein secretion of *CXCL9* on cultured macrophages stimulated with cytokines for 48 h (Figure 5E). Secretion of *CXCL9* was not significantly increased by $LT\alpha_1\beta_2$, LIGHT or TNF. However, compared to the other two cytokines, TNF induced a discrete increase in *CXCL9* secretion in half of the donors tested, suggesting high variability in the ability to respond to TNF among individuals, a trend that we also observed in response to low doses of LPS and that has been previously documented²³. Remarkably, combining TNF with low doses of LPS had the highest effect on *CXCL9* secretion while maintaining *PLA2G2D* expression on macrophages (Figure 5F) suggesting that the presence of both TNF as well as LPS can drive the *PLA2G2D CXCL9* phenotype we found to be characteristically associated with GA.

In summary, we show both in patients' samples and *in vitro* that the *PLA2G2D* and *SPP1* macrophage programs are independent and mutually exclusive. In addition, we demonstrate that $PLA2G2D^+$ macrophages, which are driven by lymphoid tissue inducing signals, respond to TNF and/or low doses of LPS to produce *CXCL9* while preserving *PLA2G2D* expression. This hybrid macrophage state appears enriched in GA and may contribute to their formation by attracting activated CXCR3⁺ T cells in lymphoid rich tissues.

DISCUSSION

Expansion of gut associated lymphoid tissues, including ILFs, is linked to persistent inflammation in IBD and may contribute to amplification of adaptive immune responses². In contrast to ILFs, GA are generally seen as containment structures that emerge when antigen clearance fails. Despite the well-described association, the pathogenesis and clinical implications of ILF expansion in IBD and GA formation in CD remain unknown. A recent study in cutaneous sarcoidosis, a non-infectious granulomatous disease, suggested a link between aberrant ILF formation and GA development¹³. Here, we integrate single-cell and spatial transcriptomics from healthy and IBD intestine with *in vitro* assays and identify a mechanistic axis that may connect lymphoid tissue induction and GA organization through LT β R pathway ligands (LT $\alpha_1\beta_2$ and LIGHT) and TNF.

In homeostasis, ILFs are tightly organized niches that support IgA responses and mucosal tolerance¹. As we show here, IBD ILFs are characterized by an increase in the abundance of IgG plasma cells and inflammatory fibroblasts. In addition, ILFs in inflamed intestine showed a marked downregulation in *CCL21* expression. *CCL21* and *CCL19* are CCR7 ligands found on endothelial cells and/or FRCs in secondary lymphoid tissues, providing essential signals for naïve lymphocyte recruitment to secondary lymphoid organs, and for positioning of T cells and DCs within lymphoid organs²⁴. A marked decrease in *CCL21* expression has also been reported in lymph nodes during immune responses²⁵ and in those draining some tumors²⁶. Lower *CCL21* expression may result in altered lymphoid chemotaxis in chronic inflamed tissues potentially in favor of effector or memory cell reactivation instead of primary immune activation, thus providing a mechanism for these lymphoid structures to contribute to disease perpetuation.

In addition, by combining scRNA-seq with spatial transcriptomics, we captured a macrophage subset not previously described in human gut. These cells, characterized by expression of *PLA2G2D*, were highly enriched in intestinal ILFs and CD-associated GA. *PLA2G2D* is a secreted phospholipase that hydrolyzes membrane phospholipids to produce bioactive lipids^{27, 28}. Data in mice showed *Pla2g2d* predominant expression in peripheral lymphoid organs and dependent on lymphoid-inducing signals²⁹, indirectly supporting the association of the *Pla2g2d* program to the lymphoid tissue environment. Indeed, murine myeloid cells expressing *Pla2g2d* were found in lymphoid tissues and reported to associate with protective responses through mobilizing pro-resolving lipid mediators^{28, 30}. More recently, expression of *Pla2g2d* in macrophages was shown to protect against obesity-associated metabolic disorders through its ability to provide ω 3 polyunsaturated fatty acids³¹ and to contribute to poor outcomes in response to viral infection in aged mice^{32, 33}. In contrast to murine data, we found no previous evidence of the association of *PLA2G2D*⁺ macrophages to gut-associated lymphoid tissues (GALT), or other lymphoid tissues in human. A study published while preparing this manuscript has shown higher *PLA2G2D* expression on DCs and macrophages in lymph nodes draining melanoma in patients with metastatic recurrence³⁴. Importantly, *Pla2g2d* was also shown in murine models to control tumor growth by inhibiting T cell responses. A published scRNA-seq dataset of ileal and colonic human biopsies including 136 samples captured a small cluster of *PLA2G2D*⁺ macrophages as well³⁵, emphasizing the

need for larger datasets to detect rare populations using scRNA-seq. The authors, however, did not provide any further characterization of this population. Remarkably, compared to other tissues, including the gut mucosa and submucosa³⁶, little is known about the macrophage states that are present in GALT². In non-intestinal lymphoid tissue, a population of CD169⁺ (SIGLEC1) macrophages are prominently found in the subcapsular sinus of lymph nodes and in the marginal zone of the spleen and play a role in antigen capture and immune responses at the entry site of secondary lymphoid organs¹⁸. In human tonsils, monocyte-derived macrophage subsets were distinguished based on their expression of CD36¹⁹. However, none of those resembles the PLA2G2D⁺ macrophage subset we describe here, which lacked expression of *CD169* mRNA and did not localize in marginal zones of the follicles. In contrast, PLA2G2D⁺ macrophages were found in resting gut lymphoid aggregates and germinal centers in the inflamed intestine. According to the literature, another macrophage population known to reside in germinal centers is the tingible body macrophage (TBM), a professional scavenger specialized in the clearance of apoptotic B cells³⁷. Based on their localization, particularly within germinal centers in inflamed tissues, PLA2G2D⁺ macrophages could represent TBMs, and we cannot rule out the possibility that they do contribute to the clearance of apoptotic cells.

Based primarily on its compartmentalization within ILF and TLS, we hypothesized that signals that induce lymphoid tissue organization⁵, including LT β R ligands LT $\alpha_1\beta_2$ and LIGHT, as well as TNF³⁸, they may drive PLA2G2D⁺ macrophage differentiation in these tissues. Our results strongly suggest that all 3 cytokines can imprint macrophages with an ILF-associated signature characterized by expression of *PLA2G2D*, a marker we found conserved across homeostatic and inflammatory ILFs. Critically, cell types associated with lymphoid tissues, including FRCs and PLA2G2D⁺ macrophages, were abundant in GA as well, but absent from non-lymphoid inflammatory lesions (e.g., mucosal ulcers/fissures), strongly supporting the hypothesis that GA emerge from previously existing lymphoid-rich structures sharing mechanisms of formation with ILFs. Indeed, spatial mapping confirmed that GA frequently appeared adjacent to regions of prominent ILF/TLS expansion as previously suggested^{39, 40} and even partially embedded within these lymphoid aggregates. In that sense, there exists ample evidence supporting the role of lymphotoxins in sustaining antimicrobial GA function. LT $\alpha_1\beta_2$ signaling via the LT β R on macrophages is essential to control infections to intracellular pathogens in mice, through activation of the anti-microbicidal activity of macrophages in GA⁴¹. Besides LT $\alpha_1\beta_2$, TNF plays an essential role as well in granulomatous responses. Indeed, TNF blockade increases the risk of reactivating latent tuberculosis in patients⁴². This assumes that TNF acts on macrophages inducing a proinflammatory M1-like phenotype that orchestrates T cell responses within GA. Interestingly, evidence in mice shows that both the LT β R and TNFR1 pathways are non-redundant in their protective roles against *Mycobacterium bovis* BCG infection, and their inhibition independently blocks the formation of GA⁴³. Our data suggest that while LT $\alpha_1\beta_2$, LIGHT and TNF induce *PLA2G2D* expression on macrophages, TNF also upregulates expression of other molecules previously shown to be expressed by GA, including CXCL9⁴⁴, S100A8 and MT1X⁴⁵, thus driving the phenotype of a population of macrophages found in GA. TNF has also been

implicated in epithelioid transformation and even fusion into multinucleated giant cells, a well-known feature of GA. An earlier study, however, showed the importance of TNF in formation of epithelioid cells at the core of GA, rather than a direct effect of TNF on cell fusion⁴⁶. We show that giant cells found in CD GA expressed *SPP1*, in agreement with animal models^{47, 48} and human pulmonary GA⁴⁹, but they did not express *CXCL9* or *PLA2G2D*. Indeed, *SPP1* was inhibited by TNF and driven instead by IL-1 β or hypoxia, supporting the requirement for highly compartmentalized cytokine niches to support GA formation.

Our results offer a framework for how overlapping cytokine pathways can generate distinct tissue outcomes. While LT $\alpha_1\beta_2$ and LIGHT support an ILF-associated *PLA2G2D* program, TNF uniquely induced expression of both *PLA2G2D* and *CXCL9* *in vitro*, together with antimicrobial genes. This provides a mechanistic route by which TNF alone or in combination with low doses of LPS, can promote GA organization by generating macrophages optimized for effector T cell recruitment and antimicrobial activation, consistent with the well-established requirement for TNF in GA maintenance and host defense. In tuberculosis, GA develop hypoxic cores as they progress toward necrosis, with oxygen tension decreasing toward the center⁵⁰. Indeed, hypoxia, but not TNF, at the core of these structures emerges as a potential driver of the *SPP1*⁺ macrophage subsets which we show interact primarily with inflammatory and profibrotic fibroblasts at the core of the GA. We hypothesize *SPP1*⁺ macrophage interactions with IAFs could contribute to the highly fibrotic nature of GA. Indeed, hypoxia-induced *SPP1*-expressing macrophages have been shown to interact with fibroblasts and induce their activation, included expression of *ACTA2*, *CTGF*, collagen I, collagen III, and *FN1* at both mRNA and protein levels⁵¹. In line with this, our data supports a role for the stromal compartment as an additional determinant of GA formation: IAFs were present in IBD-associated ILFs and expanded within GA in CD, with prominent interaction networks connecting IAFs to *SPP1*⁺ macrophages. These findings nominate a stromal–myeloid axis that may reinforce lesion compartmentalization, matrix remodeling, and pro-fibrotic trajectories.

However, our study is not without limitations. First, our conclusions although confirmed using different approaches are based on the analysis of a few cases and will need to be further validated in larger cohorts. In addition, proving causality is inherently challenging in human studies. While we identify common signals between lymphoid tissue induction and GA formation, we cannot demonstrate that there is a direct or causal link between TLS formation and GA in CD. Indeed, TLS are common in UC, where GA formation is not detected. Hence, we hypothesize that variability in responses (i.e. induction of *CXCL9* in response to TNF) may predispose to GA formation. This, however, remains untested.

Overall, our study supports a model in which lymphoid tissue inductive cues establish specialized ILF macrophage programs. In that context, chronic inflammatory signals (TNF, IL-1 β , hypoxia) and bacterial signals could repurpose these programs to enable GA formation and persistence. More broadly, our work illustrates how spatially resolved transcriptomics can reveal rare, niche-restricted immune states and assign them to specific tissue microenvironments and instructive signals. Defining how lymphoid remodeling intersects with granulomatous inflammation may help explain heterogeneity in CD pathology

and suggests distinct intervention points targeting LT β R- and TNF-driven macrophage programs and their stromal partners.

METHODS

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Human ethics and sample collection

The study protocol adhered to the principles of the Declaration of Helsinki. Samples from IBD patients (CD and UC) and non-IBD controls were included in the study. All participants provided written informed consent to participate in the study. The studies were approved by the Institutional Review Boards at Hospital Clínic Barcelona and Hospital Mútua de Terrassa. Study codes HCB/2018/1062, HCB/2022/0125, HCB/2019/0714, HCB/2020/0035 and HCB/2016/0389 and the Hospital Mutua de Terrassa (CI201901).

Patients' characteristics including sex, gender and age are included in Table S1. Sex was not considered as a biological variable; therefore, patients of both sexes were included.

Endoscopic colonic biopsies (4-8 per patient) were collected from HC, CD and UC patients during routine endoscopies performed as standard of care at the Hospital Clinic (Barcelona, Spain) or at the Hospital Mutua de Terrassa (Terrassa, Spain). Healthy controls were defined as individuals undergoing colorectal cancer screening with no dysplasia or polyps observed during the endoscopic procedure. Surgical pieces were taken from discarded resection tissue collected as part of the operating procedure.

Peripheral blood was obtained from buffy coats (Banc de Sang i Teixits, Barcelona, Spain) of healthy volunteers.

Single-cell RNA-seq

Colonic cell isolation for scRNA-seq

Biopsies were processed within 1 hour of collection. After washing biopsies with cold Hank's Balanced Salt Solution (HBSS) (Gibco, MA, USA) samples were processed as previously described¹⁴. Briefly, biopsies were mechanically and enzymatically digested using 0.5 Wünsch units/ml LiberaseTM (Roche) and 10 μ g/mL DNase I (Sigma) for 1 hour at 37 °C and 250rpm agitation. The cell suspension was filtered to obtain clean single-cell suspensions. Cells were counted and transferred to the single-cell genomics platform.

10x library preparation and sequencing

Following digestion, the 10x Genomics 3' mRNA single-cell method was used. Around 7000 cells were loaded onto the Chromium10x Genomics platform (10x Genomics, CA, USA) following manufacturer's instructions. Then, gel beads-in-emulsion (GEMs) (10x Genomics, CA, USA), barcoding, and reverse transcription were carried out using the Chromium Single Cell 3' and Chromium Single Cell V(D)J Reagent Kits (10x Genomics, CA, EEU) (user guide, no. CG000086). Full-length, barcoded cDNA was amplified by PCR using Nextera® primers (Illumina, CA, USA). Libraries were sequenced on the Illumina HiSeq 2500 platform, Illumina HiSeq 4000 platform or Illumina NovaSeq 6000 platform.

Data curation and mapping

For each sample, fastq files were processed using STARsolo pipeline v1.0⁵² using the Cell Ranger 2020-A reference transcriptome and a tailored set of parameters⁵³. For UMI deduplication, barcode matching, and adapter trimming: `--soloUMI dedup 1MM_CR, --soloCBmatchWLtype 1MM_multi_Nbase_pseudocounts, --soloUMI filtering MultiGeneUMI_CR, --clipAdapterType CellRanger4,` and `--outFilterScoreMin 30`. To replicate the EmptyDrops algorithm found in CellRanger, we used `--soloCellFilter EmptyDrops_CR`. To produce exon-only, unspliced (pre-mRNA), and RNA velocity count matrices, the `--soloFeatures Gene GeneFull Velocity` options were applied.

Cell type identification

We merged the count matrices retrieved from STARsolo using the function *merge* from the SeuratObject R package (v5.3.0). Quality control included doublet assessment using scDbtFinder (v1.16.0), also, cells that expressed fewer than 100 unique genes or had a high mitochondrial content (>65%) were removed. Data were then log-normalized; highly variable genes were identified, and data were scaled using Seurat (v5). Principal component analysis (PCA) and dimensionality reduction were performed by applying the Uniform Manifold Approximation and Projection (UMAP). After clustering, we used *FindAllMarkers()* function using `min.pct = 0.25` and `logfc.threshold = 0.25`. The clusters obtained were manually categorized into the five main cell types found in the gut mucosa: Epithelial cells, Lymphoid cells (T cells and innate lymphoid cells), Myeloid cells, Stromal cells, and B and plasma cells.

Each major cell type category was re-processed. Immunoglobulin genes were removed from all the main cell types but B and plasma cell ones to reduce background noise. Then, for all the cell types except the Epithelial ones, subsets were filtered using 25% mitochondrial gene expression. Within each main lineage, re-clustering was performed, and cell types were manually annotated based on gene expression profiles.

To identify the different macrophage populations, the macrophage-annotated cells from the myeloid compartment were subsetted, followed by independent normalization, scaling, principal component analysis, clustering, and dimensionality reduction. Cluster identity was assigned based on the expression of the top markers of each cluster.

Batch correction

To correct batch effect across samples, we applied *RunHarmony* function from Harmony (v 0.1.0), with the optimal number of principal components found for each subset as latent space and the sample of origin as batch label. After Harmony integration, we re-ran Seurat's workflow for each object, followed by UMAP generation using default settings onto Harmony-corrected space.

CosMx-SMI

Study cohort and data acquisition

The CosMx™ platform [CosMx™ Human Universal Cell Characterization RNA Panel (1000-plex) along with 20 custom genes; Nanostring, USA] was used to analyze nine FFPE samples, comprising three non-IBD healthy controls, three Crohn's disease (CD) samples, and three ulcerative colitis (UC) samples. An average of 19 fields of view (FOVs) were examined per sample, yielding a total of 171 FOVs across the dataset. Morphological visualization was performed using the following cell surface markers: B2M/CD298, PanCK, CD45, CD3 antibodies, and DAPI. Detailed information regarding sample preparation and instrument settings can be found in the dataset's initial publication by Garrido-Trigo et al. (2024) (GSE234713).

Cell segmentation

Here, we applied an improved segmentation approach that provided greater resolution. The original segmentation was generated using Cellpose (v1) parameters as described by Shanshan He et al. (2022). The updated segmentation facilitated by Nanostring utilized the same parameters but with the enhanced Cellpose (v2) algorithm, resulting in a more refined segmentation and a higher cell count. Specifically, the new segmentation identified 583,974 raw cells, compared to the 482,277 raw cells detected in the original segmentation.

Quality filtering and data preprocessing

Quality-control thresholds were based on the CosMx SMI Data Analysis User Manual (MAN-10164-04). We flagged cells that met any of the following criteria: fewer than 25 counts, more than 10% of total counts attributed to negative probes, over 10 counts of negative probes, fewer than 10 detected features, a count distribution where $(\text{total counts}) / (\text{number of detected genes}) \leq 1$, or areas identified as outliers using Grubb's test with a p-value of 0.01 (outliers v.0.15). Flagged cells were excluded from downstream analysis as they were considered artifacts. After quality control, 538,992 cells ($\approx 92.3\%$ of the dataset) remained for analysis.

Normalization and dimensionality reduction

For normalization, we applied *SCTransform* (Seurat v5.2.1). Next, we conducted principal component analysis (PCA) and examined the elbow plot to assess the variance explained by each PC. We then selected the first 50 PCs to construct the k-nearest neighbor graph (k-NN) using a k of 20 and performed dimensionality reduction via UMAP.

Annotation

Annotation was conducted using 92 single-cell RNA-seq samples. Immunoglobulin G (IgG) genes were excluded due to their high expression across all samples, mirroring the patterns observed in single-cell RNA-seq data. We performed supervised clustering with the *insitutypeML* function (InSituType v1.0.0). Initially, we classified major cellular groups — T cells, epithelium, myeloid, plasma cells, and stromal cells — retaining only cells with a classification probability of $\geq 90\%$ to ensure high confidence. Subsequently, we applied the same analysis to each subset individually to obtain a more refined population structure of 48 cell types. Finally, we extracted marker genes for each population to validate the annotation within the 1k gene panel of CosMx-SMI.

Neighborhood analysis

We adapted our neighborhood analysis method from Cadinu et al.¹⁵, which identifies statistically recurrent neighborhoods of cells. While the original method used a different number of neighbors, we adjusted it to 70 nearest neighbors based on Euclidean distance metrics, calculated using the 2D centroid coordinates of our cells. We then assigned a numerical score to each neighbor according to its proximity, with the closest neighbor receiving a score of 70/70, the second closest a score of 69/70, and so forth. These scores formed a neighborhood composition vector for each target cell. The first 5 elements of this vector represented the sum of scores for the subset classification, while the subsequent 48 elements summed the scores for the refined cell populations. To emphasize hierarchical organization, we weighted the last 48 elements by a factor of 2.

For the detection of statistically recurrent neighborhoods, we utilized Scanpy (v.1.9.8). First, we performed PCA on the matrix of neighborhood composition vectors. We then constructed a k-nearest neighbor graph using 15 neighbors and 19 PCs, generating an UMAP with default parameters. Recurrent neighborhoods were identified using Leiden clustering with a resolution of 0.5.

The selection of the number of neighbors and Leiden resolution was based on balancing spatial detail and reproducibility across the three healthy samples, which served as the reference. Increasing the number of neighbors or decreasing the Leiden resolution highlighted broader tissue regions, reducing sensitivity to smaller spatial variations. Conversely, decreasing the number of neighbors or increasing the resolution emphasized finer structural differences. The chosen parameters effectively captured both fine spatial details and consistent neighborhood patterns in the healthy samples.

We initially identified 76 clusters and ranked them by cell count and occurrence across samples. The first 21 clusters, each containing over 6,000 cells and appearing in multiple samples, accounted for 94.4% of the dataset and were retained. Smaller, less consistent clusters were assessed for potential merging based on spatial proximity, calculated as the mean minimum Euclidean distance between clusters. Clusters were then merged with the most spatially proximate of the 21 major clusters to enhance biological interpretability.

Cell interaction analysis

We conducted cell interaction analysis using Spatially Constrained Optimal Transport Interaction Analysis (SCOTIA) ¹⁶, an optimal transport model that incorporates both spatial distance and ligand-receptor gene expression. For the ligand-receptor database, we used CellTalkDB ⁵⁴ as reference.

Cell-cell co-occurrence analysis

For each sample, we analyzed radii ranging from 100 to 1,550 pixels in steps of 50. At each radius, we constructed a KDTree from Scikit-learn (v.1.5.2) using the x and y coordinates of cell centroids and built a connection matrix to quantify interactions between cell types using Squidpy (v.1.2.3). After computing the observed co-occurrence of each cell type in the tissue, we performed 1,000 simulations in which cell-type labels were shuffled across the entire sample. For each radius, we computed the mean simulated co-occurrence value (from the 1,000 simulations), and enrichment was defined as the $\log_2$ ratio of the observed co-occurrence to the mean simulated co-occurrence.

Molecular Cartography

Sample preparation

Colonic samples were freshly frozen in Optimal Cutting Temperature (OCT) compound. Samples were placed in an OCT-containing cryomold, on top of a frozen copper block. OCT froze after 5 minutes, and the cryomold was preserved at -80°C. For Molecular Cartography (Resolve Biosciences, Belgium), sections of 10µm thickness and 7x7mm large were cut and placed on Molecular Cartography slides. A panel of 100 probes (Table S6) were selected to cover most intestinal cell types, with a particular interest on macrophages and fibroblast subtypes.

Data processing

Cell segmentation was conducted by Resolve Biosciences. Initial segmentation masks were generated using Cellpose (v2). Raw Molecular Cartography outputs were processed using a custom R pipeline. For each sample, cell boundaries were extracted from ROI files generated during image segmentation and converted into polygon coordinates using the RImageJROI (v.0.1.3) and sf (v.1.0-21) packages. Polygon centroids were computed to define cell positions. Detected transcript coordinates were imported from raw text files, and transcripts were assigned to cells based on spatial inclusion within each polygon (cell boundaries) using the *point.in.polygon* function. Gene–cell count matrices were generated by aggregating transcript counts per cell and combined with cell metadata (centroids and sample identifiers) to create Seurat objects.

Human monocyte-derived macrophages

Macrophage isolation and differentiation

Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats (Banc de Sang i Teixits, Barcelona, Spain) of healthy volunteers (n = 14) over a Lymphoprep (Nycomed Pharma) gradient according to standard procedures. Monocytes were purified from PBMCs by magnetic cell sorting using CD14 Microbeads (Miltenyi Biotec). Monocytes (> 95% CD14 + cells) were cultured at 0.5×10^6 cells/ml for 7 days in Complete Medium (RPMI medium (Gibco) supplemented with 10% FBS inactivated, 1.5% HEPES (Gibco), 1% L-glutamine (Gibco), 1% Gentamicin (Cultek), 1% antibiotic/antimycotic solution (Sigma-Aldrich) at 37 °C in a humidified atmosphere with 5% CO₂. Monocyte colony-stimulating factor (M-CSF; 10 ng/ml; ImmunoTools) was added on days 0, 2, and 5. M-CSF was chosen as the differentiation model due to its ability to generate macrophages that exhibit the highest similarity to the FOLR2⁺ macrophages identified in our dataset.

Macrophage stimulation

On day 6 of macrophage differentiation, lymphotoxin-alpha1/beta2 (LTα1β2; 100 ng/mL, R&D Systems), tumor necrosis factor (TNF; 50 ng/mL, R&D Systems), light (LIGHT; 50ng/mL, R&D Systems), interleukin 1 beta (IL1β; 12.5 ng/mL, Biolegend) were added to our culture. Cells were incubated at 37 °C in conditions of normoxia (5% CO₂, 21% O₂, 37 °C) for 24 h. At the same time, we incubated the macrophages in hypoxia conditions (5% CO₂, 1.1% O₂, 37 °C) for 24 hours, using the ESCO Incubator (CCL-170T-8 model), without any type of stimuli added.

On day 7, for subsequent analyses, cells were harvested for real-time PCR (qPCR), while supernatants were collected to perform ELISA.

Additional experiments were conducted, where macrophages were incubated first with LTα1β2 (100ng/mL, R&D Systems), TNF (50ng/mL, R&D Systems) or LIGHT (50ng/mL, R&D Systems) on day 5 of macrophage differentiations. After that, on day 6 of differentiation we included a low concentration of lipopolysaccharide (LPS; 0.01ng/ml, Sigma-Aldrich) in each condition of the culture. Finally, on day 7, cells were harvested for real-time PCR (qPCR), while supernatants were collected at 48 h to measure CXCL9 production.

RNA isolation

Macrophages were collected with Buffer RLT and β-mercaptoethanol, and RNA was isolated using a Mini Kit for RNA and protein purification (NucleoSpin RNA/Protein, Macherey-Nagel) according to the manufacturer's instructions.

Total RNA was quantified using a NanoDrop spectrophotometer (Nanodrop technologies).

Quantification of gene expression (qPCR).

Total RNA was reverse-transcribed into cDNA using a reverse transcriptase (High-Capacity cDNA Archive RT kit; Applied Biosystems). qPCR was performed on a QuantStudio™ 3 Real-Time PCR (Applied Biosystems) using TaqMan Universal PCR Master Mix (Applied Biosystems) together with predesigned,

target-specific primers for the six genes of interest: *PLA2G2D* (Hs01572938_m1), *SPP1* (Hs00959010_m1), *MMP9* (Hs00957562_m1), *CXCL9* (Hs00171065_m1), *MT1X* (Hs00745167_sH) and *S100A8* (Hs00374264_g1). TBP was used as the endogenous control (TaqMan primers and probes; Applied Biosystems).

Expression was measured as arbitrary units (AU), calculated using the $2^{-\Delta Ct}$ method, where ΔCt represents the difference between the mean Ct of the target gene and the mean Ct of the endogenous reference gene.

Cytokine measurement by an enzyme-linked immunosorbent assay (ELISA)

Detection of CXCL9 was carried out in cultured macrophage supernatants using ELISA kits for human CXCL9 (DuoSet™ ELISA, R&D Systems) according to the manufacturer's instructions.

Immunohistochemistry

IHC was performed on tissue sections from surgical resections fixed in 10% neutral buffered formalin for 48-96 h and embedded in paraffin. Deparaffinization, rehydration and epitope retrieval were performed using PT Link (Agilent) using Envision Flex Target Retrieval Solution Low pH (Dako). After incubation for 45 min in blocking solution (0.5% BSA, 20% horse serum and 0.05% Tween 20 in PBS), endogenous biotin blocking was performed with Streptavidin/Biotin Blocking Kit (Vector Labs). CD31 staining was performed overnight at 4 °C using a mouse monoclonal anti-CD31 antibody diluted 1:100 in 0.5% BSA and 0.05% Tween 20 in PBS (JC70 clone, Sigma-Aldrich). After incubation with biotinylated anti-mouse antibody (Vector Labs) for 30 min at room temperature, the Vectastain ABC-AP kit (Vector Labs) combined with DAB chromogen system (Agilent/Dako) was used for signal detection. Sections were counterstained with Mayer hematoxylin. Images were acquired using a Leica Thunder microscope and LAS X software.

Dual in situ hybridization/immunohistochemistry

Dual ISH/IHC was carried out on tissue sections from surgical resection pieces fixed in 10% neutral buffered formalin for 48-96h and paraffin embedded. Expression of target mRNAs was detected by RNAscope (Bio-Techne, CO, USA) *in situ* hybridization according to manufacturer's instructions, followed by immunohistochemistry using an anti-CD68 antibody. After deparaffinization and antigen retrieval for 15 min in a steamer, tissue sections were incubated with the RNA probes for *PLA2G2D*, *SPP1* and *CXCL9* in the HybEZ oven (Bio-Techne) for 2h at 40 °C. After the 6-step amplification and signal detection using the RNAscope™ 2.5 HD Detection Reagents-RED (Bio-Techne), sections were incubated with blocking solution (0.5% BSA, 20% horse serum and 0.05% Tween 20 in PBS), followed by endogenous biotin blocking using the Streptavidin/Biotin Blocking Kit (Vector Labs). Rabbit polyclonal anti-CD68 (Sigma-Aldrich) diluted 1:500 in 0.5% BSA and 0.05% Tween 20 in PBS was incubated overnight at 4°C, followed by incubation with biotinylated anti-rabbit antibody (Vector Labs) for 30 min at room temperature. For signal detection, the Vectastain ABC-AP kit (Vector Labs) combined with DAB

chromogen system (Agilent/Dako) was used. After counterstaining with Gill's hematoxylin and baking at 60 °C for 30 minutes, slides were mounted with ECOmount medium (Biocare Medical). Images were acquired using a Leica Thunder microscope and LAS X software.

Statistical analysis

Differential expression analysis of the spatial data was performed in Seurat using the DESeq2 method. Cells from the two selected conditions were subset, and their expression was aggregated, normalized, and scaled. Genes were classified as upregulated if their $\log_2$ fold change ($\log_2FC$) exceeded $\log_2(1.2)$ and downregulated if $\log_2FC$ was below $-\log_2(1.2)$, with $FDR < 0.05$. Cell-type abundance differences were quantified at the FOV level using the propeller method (54) with the limma (v.3.58.1) R package. This approach models proportional changes in cell populations across conditions while accounting for variability between individual FOVs. Statistical significance was determined using Benjamini–Hochberg FDR correction ($FDR < 0.05$).

For the Bulk RNA-sequencing data, we performed gene-gene correlation on the normalized expression values from GSE235236 (14). For each target gene, we performed a pairwise Pearson correlation between its expression and the rest of genes across the samples. Two-tailed p-values were obtained from the Pearson's correlation test and adjusted using Benjamini-Hochberg. Correlation analyses were performed separately for HC, CD, and UC. For pathway analysis, genes that positively correlated with target genes with a score $r > 0.7$ for both UC and CD were used for enrichment analysis using EnrichR (v 3.4) against the BioPlanet 2019 database. For each pathway, significance was calculated using Fisher's exact test and p-values were adjusted for multiple testing using Benjamini-Hochberg.

To assess the statistical significance of the monocyte-derived macrophage qRT-PCR analysis, each stimulation was compared with its baseline using a two-sided Wilcoxon rank-sum test. *P*-values were adjusted for multiple comparisons using the Benjamini-Hochberg method. ELISA results were analyzed using two-sided one-sample t-test and corrected using Holm's correction for multiple comparisons.

Declarations

Data availability

Newly generated datasets, including molecular cartography (GEO: GSE327559; reviewer code: enyfuccmdlqbfyf) and single-cell RNA-seq (GEO: GSE342016; reviewer code: grejmccafnwrran), have been deposited at GEO and are publicly available as of the date of publication. Additionally, the updated spatial segmentation analysis for the CosMx-SMI spatial transcriptomics has been deposited at Zenodo (<https://zenodo.org/records/21373748>) (reviewer: <https://zenodo.org/records/21373748?preview=1&token=eyJhbGciOiJIUzUxMiJ9.eyJpZCI6ImRjYTZiNmMyLTdiZTEtNGUyYS1iNzI1LWQ3Mjc0MTdlMTVINCI6ImRhdGEiOnt9LCJyYW5kb20iOiI2ZGRkYzJjOWY3NzhmMDZmZWUwNGQwMDRkMWlyZWVhOCJ9.Uv4l-MMPFpj3u2BPHZezzT9Pt3gzbsyPWDumasW5HrErMjSLN9g-j8uvNVPOLVa3J1VVwTdvvu0nmzlnHqEWQ>). This study also analyzes previously published, publicly

available datasets, including single-cell RNA-seq (GEO: GSE290220, GSE291125, GSE253006, GSE214695), bulk-RNA sequencing (GEO: GSE235236), and the original CosMx-SMI spatial transcriptomics data (GEO: GSE234713). All original code for the analysis and figure generation has been deposited at GitHub and is publicly available as of the date of publication at https://github.com/ibd-bcn/Granuloma_IBD. To facilitate the exploration of the results, an interactive Shiny web application is freely available at <http://ibd-server.upc.es/manuscript/granuloma>. Any additional information required about the data reported in this paper is available from the lead contact upon request.

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Authors contribution statement

MB and AS-M, performed computational analysis of scRNAseq and CosMx SMI, conceptualized the results of the manuscript, generated figures and wrote computational methods, created figures, and wrote manuscript draft. SS performed experiments, created figures, wrote methods and reviewed and edited manuscript. AG-T and EM-A performed experiments and reviewed and edited manuscript. MV, ID and VG performed and supervised experiments, wrote methods, and reviewed and edited manuscript.

SL-P and LR performed experiments. AC and ME performed histological assessments, provided human samples and reviewed and edited manuscript. MA-M and EM performed computational analysis of spatial transcriptomics data. AF-C, IO, AM-C, ME, CL and ER provide human samples and clinical information and reviewed and edited the manuscript. JP acquired funding and critically reviewed the manuscript. AS acquired funding, designed, and supervised the project, and wrote the manuscript.

Ethics declaration

Competing interests

AS has received grants from Pfizer, Roche/Genentech, AbbVie, GSK, Scipher Medicine, Alimentiv, Inc, Boehringer Ingelheim, Agomab, Takeda and Nestlé; received consulting or speaker's fees from Genentech, GSK, Boehringer Ingelheim, Pfizer, Galapagos, AdBio Partners, HotSpot Therapeutics, Alimentiv, Nestle, GoodGut; Pfizer, Takeda, Johnson & Johnson, Eli Lilly and Agomab. ER has received support for congress and conference attendance, speaker fees, research support or consulting fees from MSD, Abbvie, Ferring, Janssen, Otsuka, Pfizer, Takeda, Faes Farma, Galapagos/Alphasigma, Kern Pharma, Lilly, Falk Pharma, Johnson&Johnson, and Fresenius-Kabi. MC has received consulting or speaker's fees from Lilly. ME has received financial support for conference attendance and research support from Abbvie, Ferring, Janssen and Takeda. AMC has received financial support for conference attendance, educational activities, and research support from AbbVie, Biogen, Faes Farma, Ferring, Janssen, MSD, Pfizer, Takeda, Dr. Falk Pharma, Lilly, Tillotts, Schär and Grupo Juste. CL has received consulting fees from Boston Scientific and FujiFilm.

ME has received financial support for conference attendance and research support from Abbvie, Ferring, Janssen, Pfizer, and Takeda. The rest of the authors have no competing interests.

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Figures

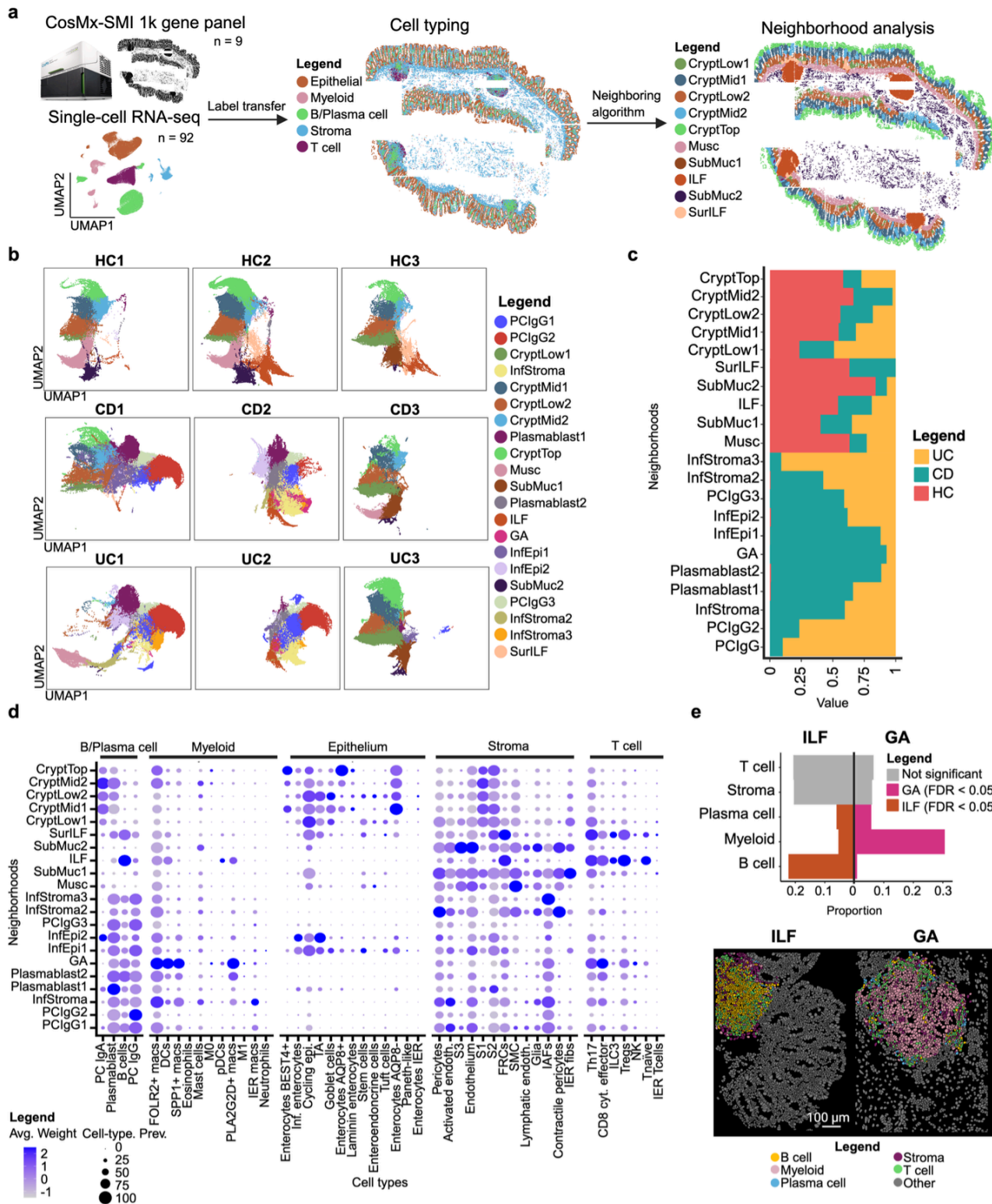


Figure 1

Neighborhood analysis of healthy and inflamed colonic sections identifies lymphoid aggregates and granuloma as distinct structures.

(a) Schematic representation of analysis workflow from scRNA-seq data ($n = 92$ samples; 6 HC, 36 UC, 50 CD) label transfer onto CosMx™ SMI dataset (3 HC, 3 UC and 3 CD) to neighborhood analysis. **(b)**

Uniform manifold approximation and projection (UMAP) of CosMx™ SMI samples (n=9), color-coded by neighborhood. **(c)** Bar plots showing the different proportions of each neighborhood per disease condition. **(d)** Dot plot detailing cell-type weights within defined spatial neighborhoods. Color intensity corresponds to the average cell-type weight, and dot size indicates cell type prevalence. **(e)** Upper: Bar plot representing the proportion of selected cell compartments in ILF and GA. Cell-type proportions were logit-transformed and compared between groups using empirical Bayes moderated t-tests. Statistical significance was determined using Benjamini–Hochberg false discovery rate (FDR) correction ($FDR < 0.05$). Lower: Representative images from CosMx SMI for ILF and GA showing the distribution of the selected cell compartments. HC: healthy control; UC: ulcerative colitis; CD: Crohn's disease; ILF: isolated lymphoid follicle; GA: granuloma.

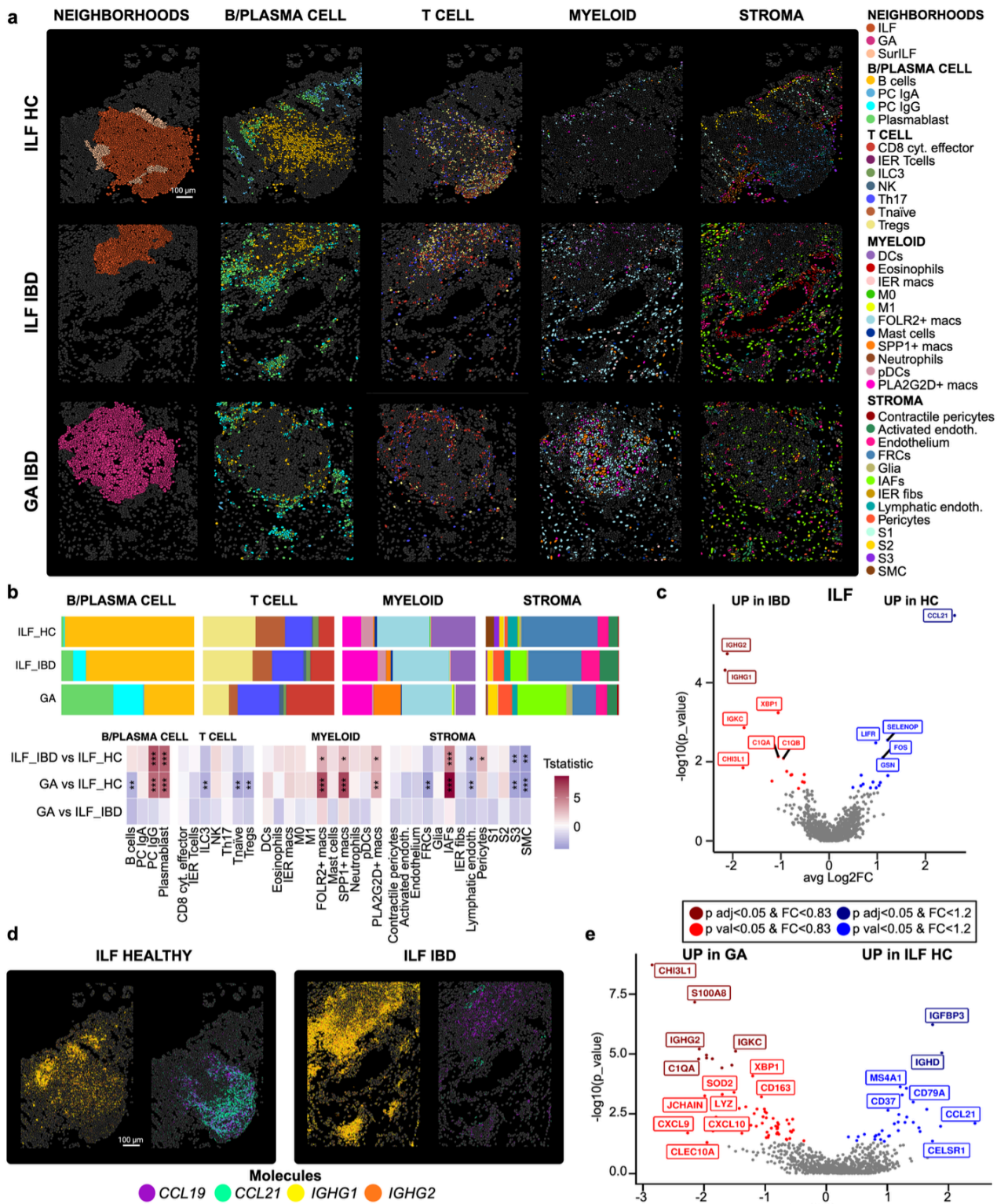


Figure 2

Comparison between healthy and IBD isolated lymphoid follicles reveals disease-associated changes in cellular composition and gene expression.

(a) Representative field-of-view images of ILF (from a HC and IBD sample) and GA (from a CD sample), colored by neighborhood (left column) or, by cell type. **(b)** Upper: Bar plots representing the cellular

composition of ILF (HC and IBD) and GA for each major lineage. Color legend is shown in panel A. Lower: T-statistic scores representing differences in the abundance of each cell type across pairwise comparisons: ILF HC vs. ILF IBD, ILF HC vs. GA, and ILF IBD vs. GA. Cell-type proportions were logit-transformed and statistically compared using empirical Bayes moderated t-tests. Moderated t-statistics were computed by dividing the difference in logit-transformed group means by an empirical Bayes-moderated standard error. Multiple testing correction was applied using the Benjamini–Hochberg false discovery rate (FDR), with significance levels indicated as * FDR < 0.05, ** FDR < 0.005, and *** FDR < 0.001. **(c, e)** Volcano plots of differentially expressed genes, where gene expression was aggregated by neighborhood and sample prior to analysis, and differential expression was analyzed using DESeq2 based on negative binomial distribution (genes were considered significantly regulated if they exhibited a Benjamini-Hochberg false discovery rate (FDR) adjusted p-value < 0.05, and a fold change (FC) > 1.2 or < 0.83), comparing: (C) IBD and HC in ILF; or (E) GA and ILF. **(d)** Selected FOV images of ILF in HC (left) and IBD (right) showing the expression of *CCL19*, *CCL21*, *IGHG1*, and *IGHG2* using CosMx SMI data. HC: healthy control; UC: ulcerative colitis; CD: Crohn's disease; IBD: inflammatory bowel disease; ILF: isolated lymphoid follicle; GA: granuloma.

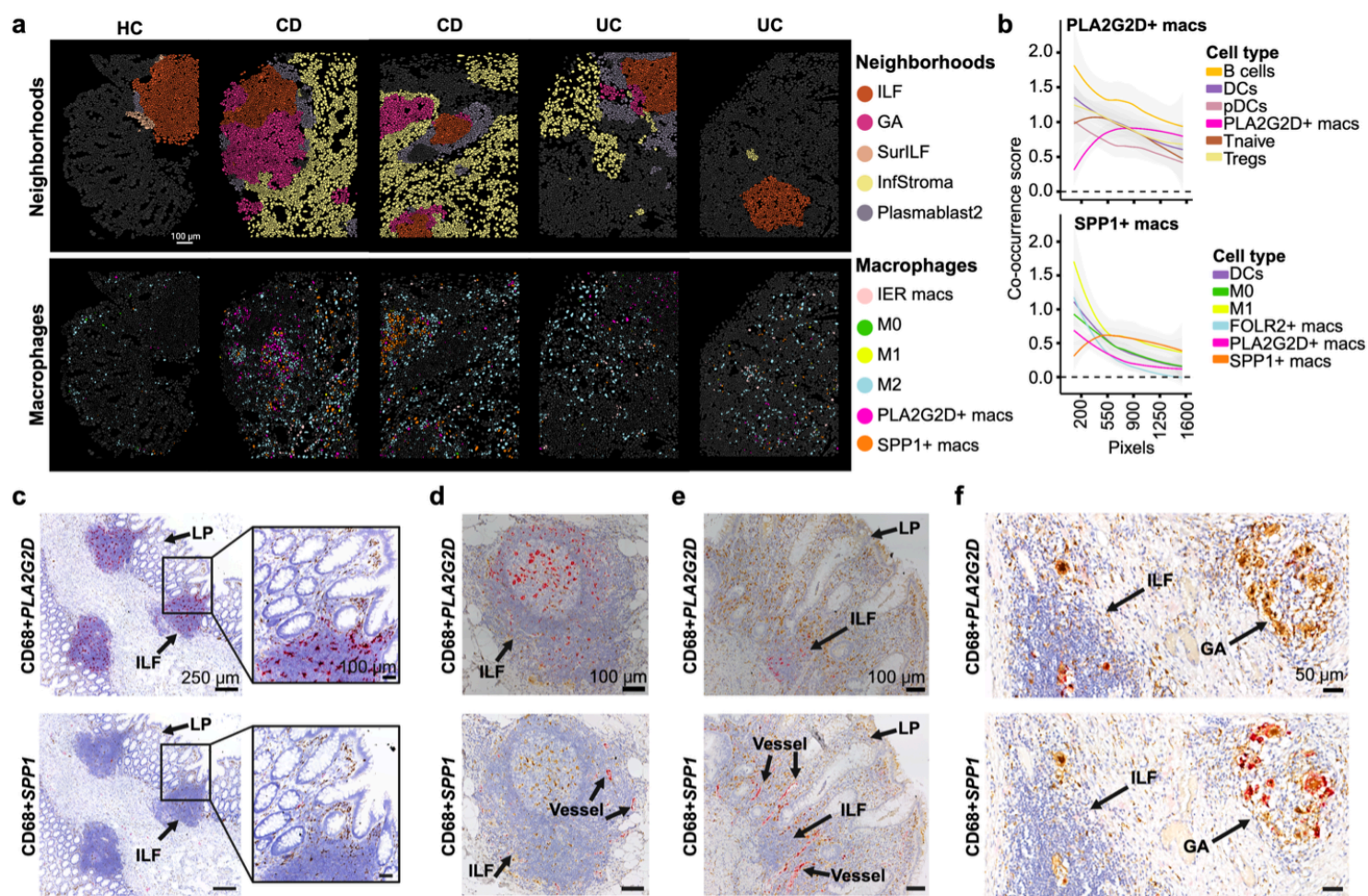


Figure 3

PLA2G2D expression identifies a macrophage subset enriched in both isolated lymphoid follicles and Crohn's disease granuloma that is distinct from *SPP1*⁺ macrophages.

(a) Selected fields of view colored by neighborhood (top row) or macrophage subset (bottom row). **(b)** Line plot depicting spatial co-occurrence between *PLA2G2D*⁺ macrophages (upper plot) or *SPP1*⁺ macrophages (lower plot), and the top 6 cell types with the highest co-occurrence scores. X-axis represents the distance radius from the reference macrophages in pixels, and Y-axis indicates the co-occurrence score. Co-occurrence scores above 0 indicate stronger spatial association. **(c-f)** Representative immunohistochemistry (anti-CD68 in brown) and in-situ hybridization (*PLA2G2D* or *SPP1*, in red) of surgical resections showing: (C) the colonic mucosa and submucosa from a HC containing 3 distinct ILFs; (D) an ILF in the colonic submucosa of a UC patient; (E) the colonic lamina propria of a UC patient; and (F) an ILF and GA in the colonic submucosa of a CD patient. HC: healthy control; UC: ulcerative colitis; CD: Crohn's disease; IBD: inflammatory bowel disease; ILF: isolated lymphoid follicle; GA: granuloma; LP: lamina propria.

(Pearson's $r > 0.7$) with *PLA2G2D* expression in bulk RNA-seq data from UC (n=26) and CD (n=43) biopsies. Dot plot showing top pathways. Dot size represents the number of overlapping genes, and dot color represents the false discovery rate (FDR) value for each pathway. **(c)** Pathway analysis of genes positively correlated (Pearson's $r > 0.7$) with *SPP1* expression in bulk RNA-seq data from UC (n=26) and CD (n=43) biopsies. Dot plot showing top pathways. Dot size represents the number of overlapping genes, and dot color represents FDR for each pathway. **(d)** Scatter plots showing the correlation between *PLA2G2D* expression and *LTB* and *LTA* across conditions in bulk biopsy RNA-seq data (n = 82); HC = 13, CD = 43, UC = 26. Correlation coefficients and FDR values were calculated using Pearson's correlation test adjusted by BH. **(e)** Expression of *PLA2G2D*, *SPP1*, and *MMP9* determined by qRT-PCR on macrophages cultured under different conditions. M-CSF-monocyte-derived macrophages from HC (n=9-14) were stimulated with $LT\alpha 1\beta 2$, LIGHT, TNF, IL-1 β or cultured under hypoxic conditions (1% O₂) for 24 h. Data are shown as fold change (FC) comparing gene expression in stimulated versus vehicle conditions. Macrophages cultured under hypoxic conditions were compared to paired normoxic cultures. Each stimulation was compared with its baseline condition using a two-sided Wilcoxon rank-sum test. P-values were adjusted for multiple comparisons using the Benjamini-Hochberg method. Significance is marked as * < 0.05, ** < 0.01, and *** < 0.001. **(f)** Chord plot visualizing high-confidence SCOTIA-inferred ligand-receptor links (likelihood > 0.6) involving top: *PLA2G2D*⁺ macrophages, middle: *SPP1*⁺ macrophages, and bottom: *FOLR2*⁺ and M0 macrophages in ILF from CD and GA, functioning as either source or target cells. HC: healthy control; UC: ulcerative colitis; CD: Crohn's disease; IBD: inflammatory bowel disease; ILF: isolated lymphoid follicle; GA: granuloma.

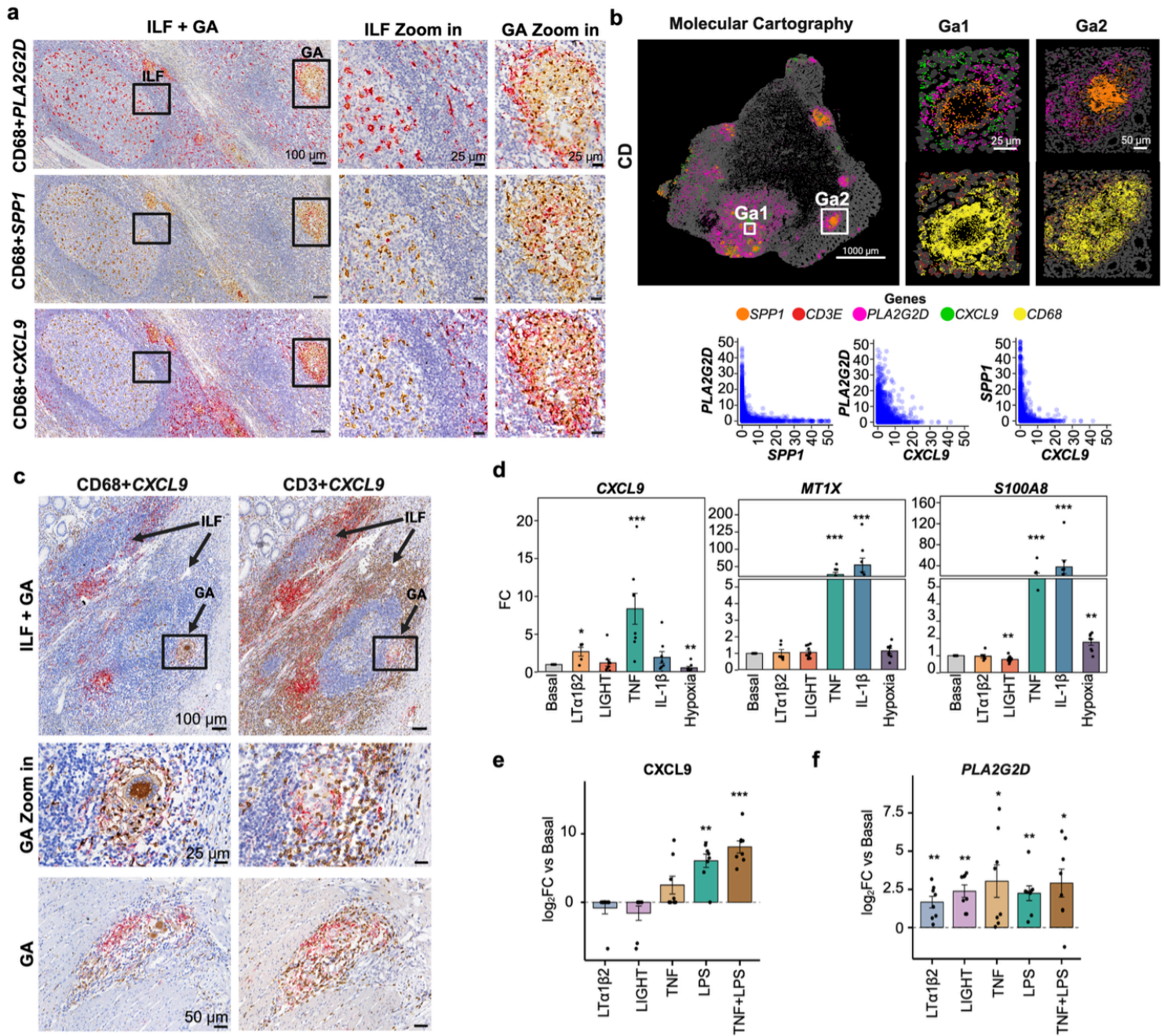


Figure 5

PLA2G2D⁺ macrophages secrete *CXCL9* in response to TNF which can be enhanced by bacterial signals.

(a) Representative immunohistochemistry (anti-CD68 in brown) and in-situ hybridization (*PLA2G2D*, *SPP1* or *CXCL9*, in red) of surgical resections showing ILFs and GA in the submucosa of a CD surgical resection. **(b)** Molecular Cartography images of a CD sample with abundant GAs. Top left: Whole-tissue section showing the expression of *SPP1*, *PLA2G2D*, and *CXCL9*. Top right: Close-up crops of GAs, depicting the localized expression of *SPP1*, *PLA2G2D*, and *CXCL9*, and the bottom depicting *CD3E* and *CD68*. Bottom: Scatter plots displaying raw transcript counts for selected gene pairs to highlight co-expression along defined cellular boundaries. **(c)** Representative immunohistochemistry (anti-CD3 in

brown) and in-situ hybridization (*CXCL9*, in red) of surgical resections showing ILFs and GA in the submucosa of two CD patients. **(d)** Expression of *CXCL9*, *MT1X*, and *S100A8* determined by qRT-PCR on macrophages cultured under different conditions. M-CSF-monocyte-derived macrophages from HC (n=9-14) were stimulated with LT α 1 β 2, LIGHT, TNF, IL-1 β or cultured under hypoxic conditions (1% O₂) for 24 h. Data is shown as fold change (FC) comparing gene expression in stimulated versus vehicle conditions. Macrophages cultured under hypoxic conditions were compared to paired normoxic cultures using a two-sided Wilcoxon rank-sum test. P-values were adjusted for multiple comparisons using the Benjamini-Hochberg method. Significance is indicated as * < 0.05, ** < 0.01, and *** < 0.001. **(e)** Secretion of *CXCL9* determined by ELISA in culture supernatants (left panel) or **(f)** expression of *PLA2G2D* determined by qRT-PCR of macrophages cultured under different conditions. M-CSF-monocyte-derived macrophages from HC (n=8) were stimulated with LT α 1 β 2, LIGHT, TNF, during 48h. Stimulation with low dose LPS (0.01ng/ml) was performed where indicated during the last 24 h of the 48h culture. Data is shown as fold change (FC) comparing gene expression in stimulated versus vehicle conditions. Statistical significance was assessed using two-sided one-sample t-test and corrected using Holm's correction for multiple comparisons. Significance is indicated as * < 0.05, ** < 0.01, *** < 0.001, and **** < 0.0001. HC: healthy control; CD: Crohn's disease; ILF: isolated lymphoid follicle; GA: granuloma.

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