Extracellular matrix-associated cytokines regulate CD4^{+} effector T-cell responses in the human intestinal mucosa

KR Huff[1], LN Akhtar[2], AL Fox[1], JA Cannon[3], PD Smith[1,4] and LE Smythies[1]

Extracellular matrix (stroma) regulation of mucosal T-cell function is incompletely understood. In this study, we uncovered a role for intestinal stromal products in the innate regulation of effector T cells. Stroma-conditioned media (S-CM) derived from the normal human intestinal stroma (transforming growth factor-β (TGF-β)^{hi}/interleukin (IL)-6^{lo}/IL-1β^{lo}) significantly downregulated T-cell proliferation and interferon-γ (IFN-γ) production compared with S-CM derived from the inflamed Crohn's mucosa (TGF-β^{hi}/IL-6^{hi}/IL-1β^{hi}). Antibody neutralization studies showed that TGF-β in normal S-CM inhibited T-cell proliferation and IFN-γ production, whereas IL-6 plus IL-1β in Crohn's S-CM promoted T-cell proliferation, and IL-1β alone promoted IFN-γ and IL-17 release. Importantly, normal S-CM inhibited T-bet expression, whereas Crohn's S-CM activated signal transducer and activator of transcription 3, suggesting that discordant T-cell responses are regulated at the transcription factor and signaling levels. These findings implicate stromal TGF-β in the downregulation of T-cell 2responses in the normal intestinal mucosa, and stromal IL-6 and IL-1β in the promotion of Th1 and Th17 responses in the inflamed Crohn's mucosa, suggesting an innate regulatory function for the intestinal extracellular matrix.

INTRODUCTION

Mucosal homeostasis—the finely tuned balance between tolerance to commensal bacteria and inflammatory responses to pathogens—is maintained through a network of complementary regulatory processes. To begin to dissect this network in the human intestinal mucosa, we have shown that intestinal extracellular matrix (stroma)-associated transforming growth factor-β (TGF-β) promotes the recruitment of proinflammatory monocytes and mediates monocyte differentiation into noninflammatory intestinal macrophages, thereby contributing to the absence of mucosal inflammation in the normal human small intestine.[1–4] T cells in the normal intestinal mucosa also are downregulated, reflected in their reduced capacity to proliferate and produce interferon-γ (IFN-γ) compared with circulating blood T cells,[5–7] but the homeostatic mechanisms responsible for T-cell downregulation in the normal mucosa are not well understood. In Crohn's disease mucosa, inappropriate proinflammatory Th1 and Th17 responses to commensal bacteria[8] seem to be due, in part, to reduced TGF-β signaling.[9] Factors that contribute to this impaired TGF-β signaling may include interleukin (IL)-6 and IL-1β.[10,11] These cytokines, abundantly present in the inflamed Crohn's disease mucosa but not in the normal mucosa,[8] promote Th1 responses[12,13] and together with TGF-β promote Th17 responses.[14–16] Although the source of these cytokines is presently unclear, the above findings suggest that the mucosal microenvironment in Crohn's disease is involved in the proinflammatory responses of local effector T cells.

In this study, we investigated whether factors associated with the stroma in the human intestinal mucosa regulate T cells using a novel system that recapitulates the *in vivo* exposure of newly recruited blood T cells to the lamina propria stroma. We have defined stroma as the lattice of collagen, fibronectin, and laminin, plus the cells responsible for their production, which mediate cytokine and cell adhesion, and transmit information in a bi-directional manner to local immune cells.[1,2] We report that stroma-associated cytokines, especially TGF-β, from the normal intestinal mucosa downregulate effector T-cell responses,

[1]Department of Medicine (Gastroenterology), University of Alabama at Birmingham, Birmingham, Alabama, USA. [2]Department of Cell Biology, University of Alabama at Birmingham, Birmingham, Alabama, USA. [3]Department of Surgery (Gastrointestinal), University of Alabama at Birmingham, Birmingham, Alabama, USA. [4]VA Medical Center, Birmingham, Alabama, USA. Correspondence: PD Smith (pdsmith@uab.edu) or LE Smythies (lesmy@uab.edu)

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but stroma-associated proinflammatory cytokines, namely IL-6 and IL-1β, together with TGF-β, from the inflamed Crohn's mucosa, potentiate proinflammatory effector T-cell responses. These findings identify a previously underappreciated contribution of the local extracellular matrix to the innate regulation of mucosal T cells in the normal and inflamed human intestinal mucosa.

RESULTS

Stromal factors derived from the normal intestinal mucosa downregulate T-cell responses more effectively than do stromal factors from Crohn's mucosa

To recapitulate *in vitro* the exposure of newly recruited blood T cells to the intestinal lamina propria and to determine whether stroma-associated molecules in the lamina propria regulate T cells, we cultured blood T cells in intestinal stroma-conditioned media (S-CM) and measured T-cell proliferation and IFN-γ production. Blood CD4$^+$ T cells from normal subjects preincubated with increasing concentrations of normal or Crohn's S-CM showed differential dose-dependent reductions in CD3/CD28-stimulated (**Figure 1a**) and phytohemagglutinin stimulated (**Supplementary Figure 1a** online) proliferation. Inhibition of T-cell proliferation was significantly greater for T cells cultured in normal S-CM than for T cells cultured in Crohn's S-CM at both 250 μg ml^{-1} ($P < 0.05$) and 500 μg ml^{-1} ($P < 0.05$) (**Figure 1b** and **Supplementary Figure 1b** online). Moreover, stromal inhibition of T-cell proliferation by normal S-CM was evident not only in a reduced percentage of proliferating T cells but also in a reduced number of generations of daughter cells.

Inducible T-cell production of IFN-γ, the prototypic Th1 cytokine, was also reduced in the presence of normal and Crohn's S-CM ($P < 0.05$ at each concentration) (**Figure 1c** and **Supplementary Figure 1c** online), and similar to the inhibition of proliferation, normal S-CM more potently downregulated IFN-γ production than Crohn's S-CM, reaching significance at S-CM concentrations of 250 μg ml^{-1} ($P < 0.05$) and 500 μg ml^{-1} ($P < 0.04$) (**Figure 1d**). Importantly, the percentage of T cells producing inducible IFN-γ was 5% in normal S-CM and 25% in

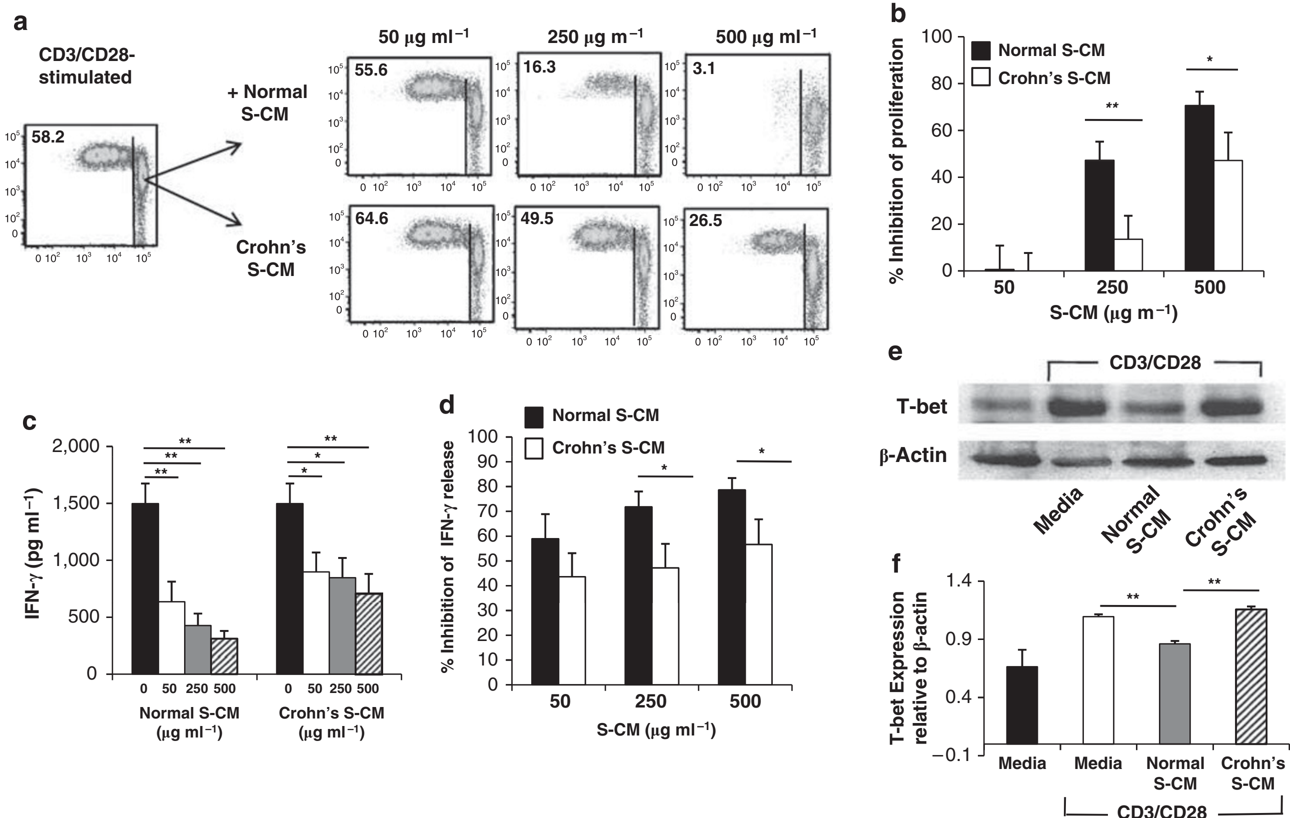

Figure 1 S-CM inhibition of CD4$^+$ T-cell responses. (**a**) CD3/CD28-stimulated T cells cultured in media alone or in the presence of a representative normal or Crohn's S-CM were analyzed on day 4 for proliferation by flow cytometry for CFSE dilution. The percentage of cells that proliferated is indicated in the top left corner of the plots. (**b**) The percentage inhibition of proliferation for CD3/CD28-stimulated T cells cultured in normal S-CM (n=7) or Crohn's S-CM (n=6). (**c**) IFN-γ release by CD3/CD28-stimulated T cells cultured for 4 days in the presence of media, normal S-CM (n=5), or Crohn's S-CM (n=6). (**d**) Percentage inhibition of IFN-γ release by CD3/CD28-stimulated T cells cultured for 4 days in the presence of normal S-CM (n=5) or Crohn's S-CM (n=6) compared with stimulated T cells cultured in media alone. (**e**) Western blot analysis of T-bet protein expression in CD3/CD28-stimulated T cells cultured 24 h in the presence of a representative normal S-CM or Crohn's S-CM (n=3 each). (**f**) Densitometric analysis of T-bet relative to β-actin (n=3). For panels **b**–**d** and **f**, error bars indicate s.e.m. *$P \leqslant 0.05$, **$P \leqslant 0.01$. CFSE, carboxyfluorescein succinimidyl ester; IFN-γ, interferon-γ; S-CM, stroma-conditioned media.

Crohn's S-CM (data not shown), indicating that Crohn's but not normal S-CM permits selective Th1 cell expansion. Moreover, T-cell expression of the Th1 transcription factor T-bet was more potently downregulated by normal S-CM than by Crohn's S-CM (**Figure 1e** and **f** and **Supplementary Figure 1d** online), indicating that stromal modulation of T-cell function was likely regulated at the transcription factor level.

We next determined whether stromal factors can similarly regulate T-cell proliferation and cytokine release in previously activated (dividing) T cells, thereby mimicking the arrival of activated T cells into the mucosa. Proliferation and IFN-γ release by T cells preactivated for 3 days with CD3/CD28 or PHA and then cultured for a further 4 days in the presence or absence of a second stimulation (CD3/CD28) was also profoundly inhibited by S-CM from the normal mucosa (**Supplementary Figures 2** and **3** online). S-CM derived from Crohn's mucosa was less efficient at inhibiting these T-cell functions than normal S-CM, consistent with our earlier findings (**Figure 1**). These findings suggest that stromal products from the normal intestinal mucosa powerfully inhibit T-cell function of both resting and stimulated T cells, and are significantly more effective at downregulating T-cell function than products from Crohn's mucosa, indicating that reduced innate stromal regulation likely contributes to the inflammatory T-cell response in Crohn's disease.

Crohn's disease intestinal stroma contains higher levels of proinflammatory cytokines than normal intestinal stroma

As cytokines have a critical role in regulating T-cell function, we analyzed S-CM generated from the normal mucosa and Crohn's mucosa for eight key regulatory cytokines. Levels of TGF-β and IL-10 in normal S-CM and Crohn's S-CM were similar, with the level of TGF-β markedly higher than that of IL-10 (**Table 1**). In contrast, levels of the proinflammatory cytokines IL-6 and IL-1β were >10-fold higher in Crohn's S-CM than in normal S-CM ($P=0.03$). Although the level of IFN-γ was higher in Crohn's S-CM than in normal S-CM, overall the level was very low (<2.3 pg ml^{-1}). Tumor necrosis factor-α was also present at barely detectable levels in S-CM derived from the normal and Crohn's mucosa. The markedly low levels of IFN-γ and tumor necrosis factor-α (as well as those of both IL-2 and IL-10) in S-CM is consistent with the absence of effector T cells and macrophages in the hematopoietic cell-depleted stroma used to generate S-CM, underscoring the relevance of S-CM as a model for the extracellular matrix. These data suggest that the loss of homeostatic downregulation in Crohn's disease mucosa is not due to lower levels of regulatory cytokines (TGF-β and IL-10) and increase the possibility that stroma-associated IL-6 and IL-1β shift the balance from tolerance to inflammation in Crohn's mucosa.

Stromal TGF-β promotes downregulation of T-cell responses

We have shown that stromal TGF-β mediates the differentiation of proinflammatory monocytes into cells with the phenotype and functional profile of noninflammatory intestinal macrophages.[2–4] Others have shown that blockade of TGF-β in mucosal biopsies leads to increased mucosal Th1 responses.[6] As TGF-β associates with the extracellular matrix[17] and is released into S-CM,[2,4] we assessed the contribution of stromal TGF-β to the downregulation of T-cell function mediated by normal S-CM. Neutralization of TGF-β in normal S-CM significantly inhibited S-CM downregulation of inducible T-cell proliferation (**Figure 2a** and **b**, and **Supplementary Figure 4a** online) and IFN-γ production (**Figure 2c** and **Supplementary Figure 4b** online) in a dose-dependent manner, thus implicating a critical role for TGF-β in normal S-CM downregulation of T-cell function. In parallel experiments, equivalent amounts of the irrelevant isotype control antibody did not affect T-cell proliferation or cytokine release (**Supplementary Figure 5a–c** online), and neutralization of TGF-β in complete media with human serum containing low levels of TGF-β also had no effect on T-cell proliferation and IFN-γ release. We next evaluated whether the level of TGF-β in S-CM was sufficient to downregulate T-cell proliferation and cytokine production. Recombinant human (rh) TGF-β alone at 50–500 pg ml^{-1} induced a dose-dependent downregulation of T-cell proliferation (**Figure 3a** and **Supplementary Figure 4c** online). However, rhTGF-β alone

Table 1 Levels of immune-modulating cytokines* in stroma-conditioned media derived from the normal mucosa and inflamed Crohn's mucosa

	Normal mucosa (n=4)		Crohn's mucosa (n=7)		
	Mean (pg ml^{-1})	s.e.m.	Mean (pg ml^{-1})	s.e.m.	P-value
TGF-β	210.00	75.00	275.00	37.00	0.40
IL-10	3.06	0.10	3.89	0.76	0.44
IL-6	6.37	1.73	255.40	70.39	0.03
IL-1β	7.14	0.37	71.40	29.00	0.03
IFN-γ	1.59	0.05	2.29	0.20	0.03
IL-17	10.00	0.70	16.00	2.00	<0.50
IL-2	2.28	0.36	4.21	1.15	0.25
TNF-α	1.18	0.36	2.88	0.65	0.04

Abbreviations: IL, interleukin; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α.
*Cytokine concentrations are standardized to 1.8 mg ml^{-1} total protein.

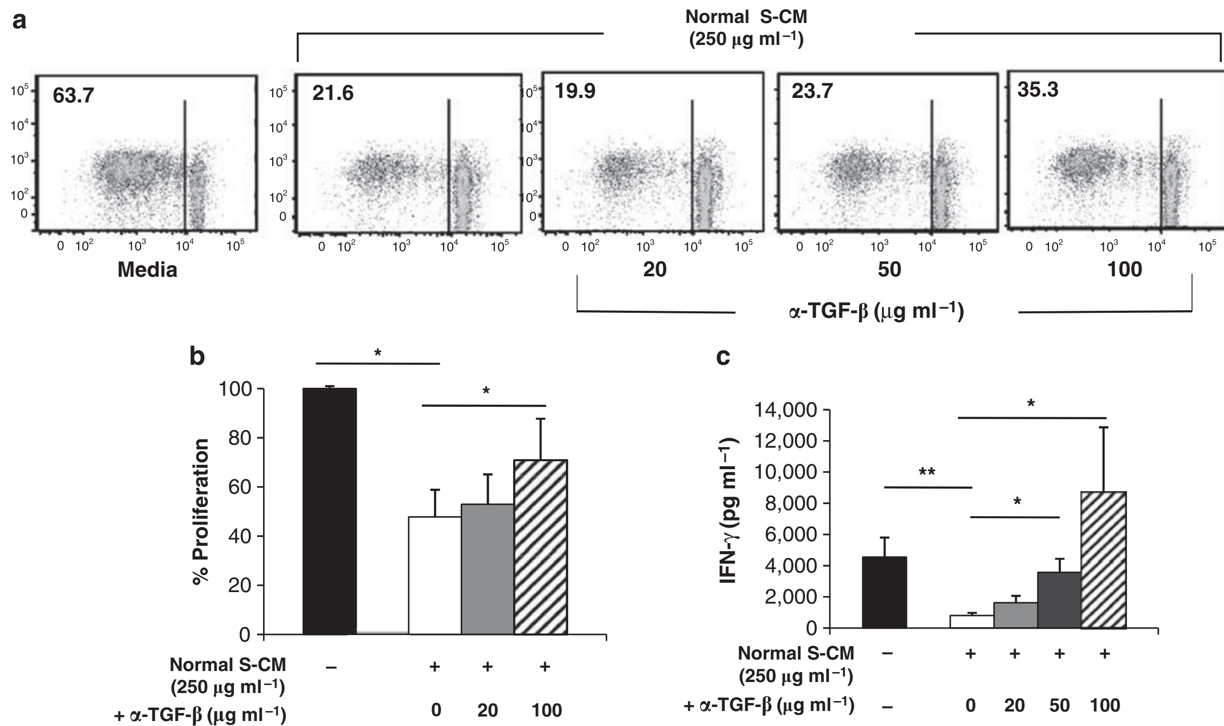

Figure 2 Neutralization of TGF-β reverses normal S-CM-mediated downregulation of CD4+ T-cell responses. (**a**) Proliferation of CD3/CD28-stimulated T cells cultured for 4 days in the presence of media or a representative normal S-CM (250 μg ml^{-1}) ± anti-TGF-β-neutralizing antibody. (**b**) Percentage proliferation of stimulated T cells cultured in the presence of normal S-CM±anti-TGF-β antibody compared with stimulated T cells cultured in media alone (n=4). (**c**) IFN-γ release by CD3/CD28-stimulated T cells cultured for 4 days in the presence of media or normal S-CM ± anti-TGF-β antibody (n=3). For panels **b** and **c**, error bars indicate s.e.m. *$P \leq 0.05$ and **$P \leq 0.01$. IFN-γ, interferon-γ; S-CM, stroma-conditioned media; TGF-β, transforming growth factor-β.

at 50 and 500 pg ml^{-1} was less effective at downregulating T-cell proliferation than normal S-CM (250 μg ml^{-1}) ($P < 0.01$ for each concentration) (**Figure 3b**). (It must be noted that 50 pg ml^{-1} rhTGF-β is equivalent to the level of TGF-β in normal S-CM at a concentration of 250 μg protein per ml (**Supplementary Table 1** online)). rhTGF-β at 50–500 pg ml^{-1} also inhibited T-cell IFN-γ release ($P < 0.05$–0.01) (**Figure 3c** and **Supplementary Figure 4d** online). These findings suggest that TGF-β is necessary for T-cell downregulation in the mucosa, although alone it is not sufficient to achieve the full downregulation of T-cell responses induced by normal S-CM.

Stromal IL-6 and IL-1β derived from Crohn's mucosa promote proinflammatory T-cell responses

Both TGF-β and IL-10 were present at similar levels in normal and Crohn's S-CM, but the levels of IL-6 and IL-1β, which also associate with extracellular matrix,[18,19] were increased in Crohn's S-CM (**Table 1**). Therefore, we investigated whether stromal IL-6 and IL-1β contribute to the reduced ability of Crohn's S-CM to inhibit T-cell responses. Preincubation of Crohn's S-CM with an optimal concentration of neutralizing antibody to IL-6, but not IL-1β, enhanced Crohn's S-CM downregulation of CD3/CD28-stimulated T-cell proliferation (**Figure 4a**). Moreover, simultaneous blockade of IL-6 and IL-1β further enhanced Crohn's S-CM downregulation of T-cell proliferation (n = 4). Furthermore, neutralization of IL-1β alone or IL-1β plus IL-6 in Crohn's S-CM significantly reduced inducible T-cell IFN-γ production ($P < 0.05$) (**Figure 4b**). These findings implicate discordant roles for IL-6 and IL-1β in the modulation of effector T-cell function in Crohn's mucosa.

As IL-6 mediates its response through signal transducer and activator of transcription 3 (STAT3) signaling, we analyzed STAT3 phosphorylation in T cells exposed to normal S-CM or Crohn's S-CM. T cells exposed to Crohn's, but not normal, S-CM phosphorylated STAT3 (**Figure 4c**). Neutralization of IL-6, or IL-6 plus IL-1β, but not IL-1β alone, in Crohn's S-CM substantially reduced STAT3 phosphorylation in T cells exposed to Crohn's S-CM, suggesting that the effect of IL-6 in Crohn's S-CM on effector T-cell proliferation and cytokine release is mediated through STAT3 activation.

Stromal factors in Crohn's disease promote Th17 responses

STAT3 activation has a critical role in the development of IL-17-producing T cells.[16] Therefore, we determined whether the discordant regulation of T-cell function by normal vs. Crohn's stromal factors extended to IL-17 secretion. In the presence of normal S-CM, CD3/CD28-stimulated T-cell production of IL-17 was reduced, although not significantly, compared with control T cells (**Figure 5a**). However, in the presence of increasing concentrations of Crohn's S-CM, T cells secreted markedly higher levels of IL-17 than did T cells cultured in media alone ($P < 0.01$), with similar amounts of IL-17 released at all concentrations

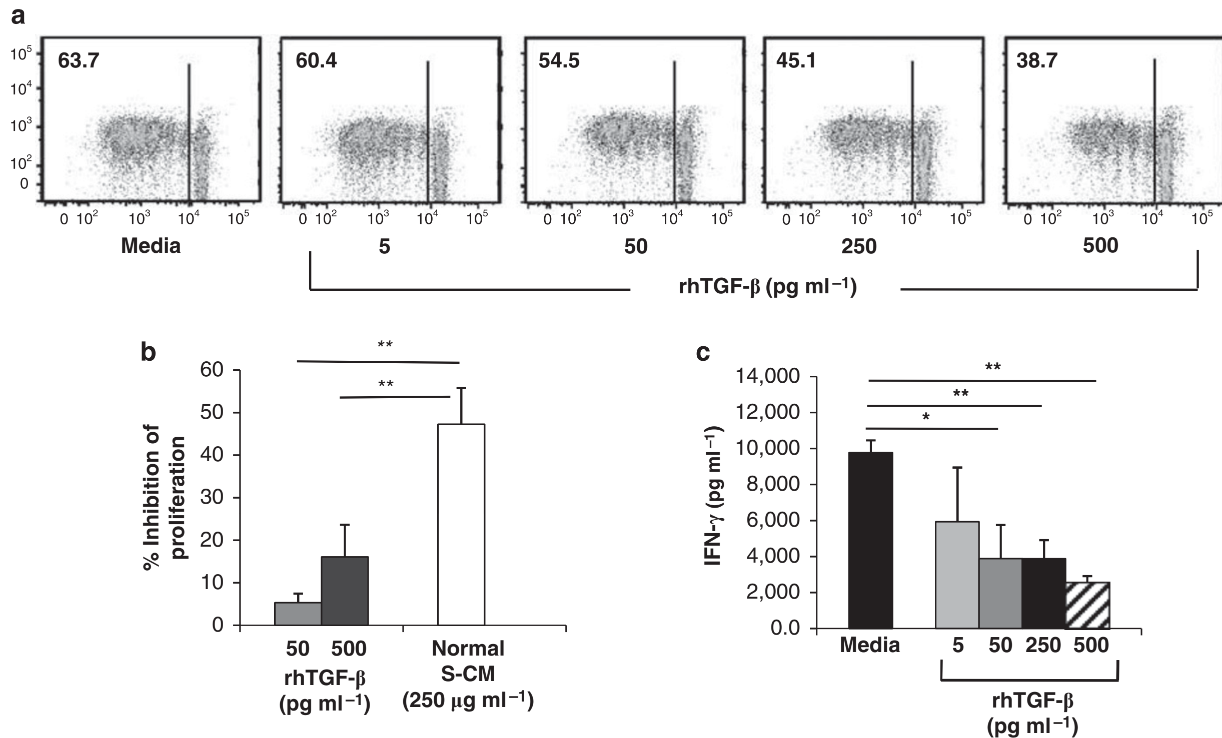

Figure 3 TGF-β promotes downregulation of CD4+ T-cell responses. (**a**) Proliferation of CD3/CD28-stimulated T cells cultured in the presence of rhTGF-β alone for 4 days in a representative experiment ($P \leqslant 0.05$ at 50, 250, and 500 pg ml^{-1} for $n=4$). (**b**) Percentage inhibition of CD3/CD28-stimulated T-cell proliferation by rhTGF-β alone ($n=4$) at 50 or 500 pg ml^{-1} compared with normal S-CM (250 μg ml^{-1}) ($n=7$). (**c**) CD3/CD28-stimulated IFN-γ release by T cells cultured in the presence rhTGF-β ($n=3$). For panels **b** and **c**, error bars indicate s.e.m. *$P \leqslant 0.05$ and **$P \leqslant 0.01$. IFN-γ, interferon-γ; S-CM, stroma-conditioned media; TGF-β, transforming growth factor-β.

of Crohn's S-CM (**Figure 5a** and inset). As the proportion of proinflammatory (IL-6 and IL-1β) to downregulatory (TGF-β) cytokines was constant at each concentration of Crohn's S-CM, these findings suggest that the relative proportion of the cytokines was more important than their absolute concentration in the regulation of IL-17 secretion in the presence of Crohn's S-CM.

We next investigated whether blockade of IL-6, IL-1β, or IL-6 plus IL-1β in Crohn's S-CM would reverse the enhanced IL-17 production by T cells. Neutralization of IL-1β, but not IL-6, in Crohn's S-CM significantly reduced IL-17 production ($P=0.04$), reaffirming the critical role of IL-1 in promoting human Th17 responses (**Figure 5b**). Furthermore, neutralization of IL-1β plus IL-6 in Crohn's S-CM reduced IL-17 production to levels similar to CD3/CD28-stimulated IL-17 released by T cells in media alone. Given the low level of IL-17 in Crohn's S-CM (16±2 pg ml^{-1}, $n=7$; **Table 1**), these results implicate T cells as the source of IL-17 and suggest that the balance between IL-1β (with IL-6) and TGF-β contributes to the pathogenic Th17 responses in Crohn's disease.

DISCUSSION

The gastrointestinal mucosa is continuously exposed to a huge array of immunostimulatory antigens and microorganisms, necessitating the evolution of mechanisms to downregulate potentially harmful inflammatory responses. The remarkable plasticity of the normal mucosal immune system of the small intestine is evident in the capacity of the system to respond to commensal bacteria with tolerance but to pathogens with an inflammatory response to contain the microorganism. However, this homeostatic regulation is disrupted in certain inflammatory processes such as Crohn's disease, when T cells respond inappropriately and exuberantly to commensal bacteria with increased proliferation and cytokine (IFN-γ and IL-17) production.[8,20–22]

To elucidate the mechanisms responsible for the hyporesponsiveness of T cells in the normal mucosa, but hyperresponsiveness in Crohn's disease mucosa, we developed a novel system that recapitulates the *in vivo* exposure of newly recruited blood T cells to the lamina propria extracellular matrix products derived from normal or inflamed Crohn's disease mucosa. Using this system, we uncovered a previously underappreciated role for extracellular matrix-associated cytokines in the regulation of T-cell responses in the intestinal mucosa. We show that the stroma from normal and Crohn's disease mucosa released similar levels of the downregulatory cytokine TGF-β and nearly undetectable levels of IL-10. However, the stroma from Crohn's disease mucosa released elevated levels of the proinflammatory cytokines IL-6 and IL-1β compared with the normal intestinal stroma. TGF-β released by the cultured normal stroma

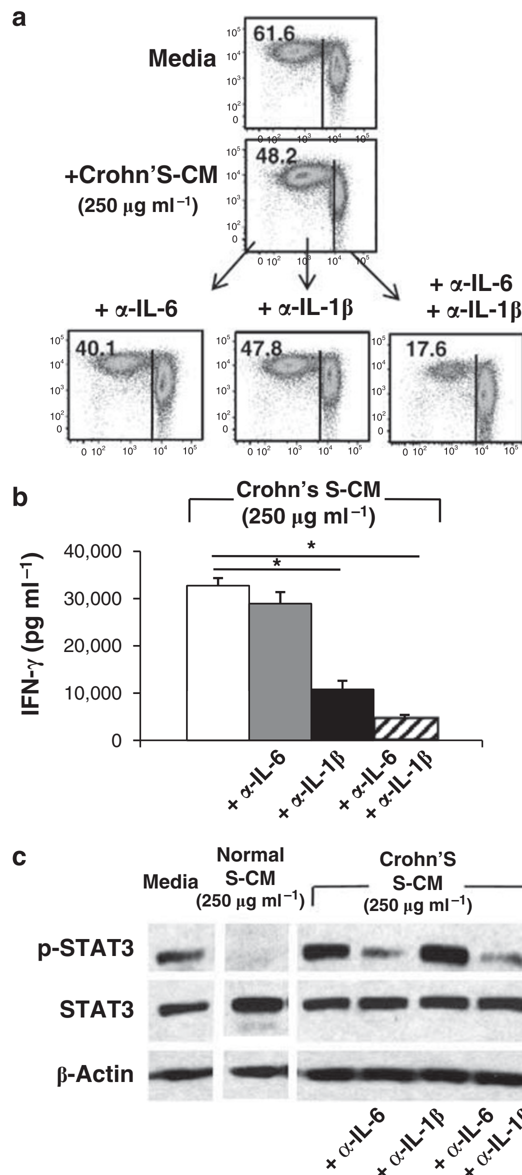

Figure 4 IL-6 and IL-1β in Crohn's S-CM promote proinflammatory CD4+ T-cell responses. (**a**) Proliferation (n=3) and (**b**) IFN-γ release (n=2; each performed in triplicate) by CD3/CD28-stimulated T cells cultured for 4 days in media or a representative Crohn's S-CM ± anti-IL-6 and/or anti-IL-1β-neutralizing antibodies (1 μg ml−1). (**c**) Western blot analysis of STAT3 and p-STAT3 protein expression by T cells exposed for 30 min to media, a representative normal S-CM or a representative Crohn's S-CM±anti-IL-6 and/or anti-IL-1β antibodies (1 μg ml−1) (n=2). For panel **b**, error bars indicate s.e.m. *P≤0.05. IFN-γ, interferon-γ; IL, interleukin; S-CM, stroma-conditioned media; STAT3, signal transducer and activator of transcription 3.

inhibited T-cell function, whereas IL-6 plus IL-1β released by Crohn's stroma promoted T-cell proliferation, and stromal IL-1β alone induced T cells to release both IFN-γ and IL-17.

Stromal products derived from the normal intestinal mucosa downregulated T-bet expression and IFN-γ production. However, Crohn's stromal products did not inhibit T-bet

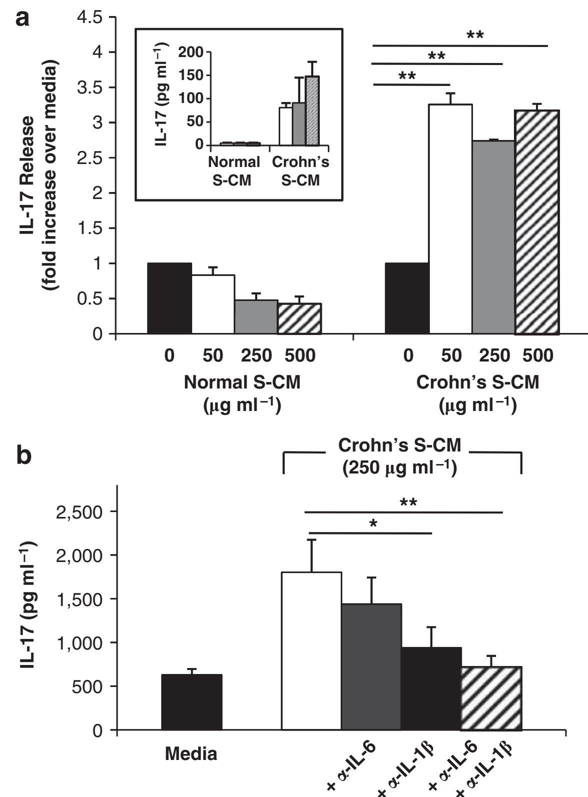

Figure 5 Crohn's S-CM promotes IL-17 release by CD4 T cells in an IL-1β-dependent manner. (**a**) IL-17 release by CD3/CD28-stimulated T cells cultured 4 days in the presence of normal S-CM or Crohn's S-CM compared with stimulated T cells cultured in media alone (n=3). Inset shows results from a representative experiment (n=3) with IL-17 release (pg ml−1) by CD3/CD28-stimulated blood T cells cultured for 4 days in the presence of normal or Crohn's S-CM. (**b**) IL-17 release by CD3/CD28-stimulated T cells cultured for 4 days in Crohn's S-CM ± anti-IL-6 antibody and/or anti-IL-1β antibodies (1 μg ml−1) (n=2, each performed in triplicate). Error bars indicate s.e.m. *P≤0.05 and **P≤0.01. IL, interleukin; S-CM, stroma-conditioned media.

expression but did reduce IFN-γ production, suggesting involvement by additional regulatory mechanisms, such as impaired accessibility of T-bet to its DNA-binding site, decreased mRNA stability, and improper protein folding and export.[23] Among the products released by Crohn's tissue stroma, IL-6 induced STAT3 phosphorylation. In this regard, STAT3 activation has a critical role in Th17 differentiation,[16] and in mouse models, IL-6 in the presence of TGF-β promotes the differentiation of Th17 cells. The differentiation of Th17 cells in humans is less well understood.[24,25] However, we show in this study that Crohn's stromal products enhanced IL-17 release from CD3/CD28-stimulated T cells in an IL-1β-dependent manner and others have reported that IL-1β enhanced secretion of IL-17 from human memory T cells.[25,26] Taken together, our results suggest that stromal factors in Crohn's disease mucosa do not differentiate T cells into Th17 cells, but more likely promote IL-17 secretion from Th17 memory cells that have entered the mucosa.

Our findings implicate a role for stromal TGF-β in the downregulation of T-cell responses in the normal intestinal mucosa,

but stromal IL-6 and IL-1β in the promotion of Th1 and Th17 responses in the inflamed Crohn's mucosa, despite the continued presence of local TGF-β. As apoptotic cells are also a potential source of TGF-β, it is important that S-CM derived from both normal and Crohn's disease mucosa had a slightly protective effect on T-cell apoptosis (data not shown), confirming that TGF-β responsible for regulating T-cell function was derived from the stroma. Moreover, the fact that intestinal stromal factors inhibit T-cell proliferation and cytokine release in preactivated T cells suggests that the mucosal microenvironment may also inhibit mucosal inflammation enhanced by newly recruited, activated T cells. Thus, these findings identify an innate regulatory function for the intestinal extracellular matrix, reflected in the ability of the intestinal stroma to regulate T-cell responses. To dissect the specific role of the stroma, we used normal blood T cells as indicator cells. Whether T cells from patients with Crohn's disease are resistant to mucosal downregulation by normal stromal factors is the subject of a separate study.

Investigative attention has recently focused on the conditioning of mucosal macrophages[1–4] and DCs.[27–32] Regarding DCs, epithelial cells produce TGF-β and transcriptionally active retinoic acid that act as cofactors to drive the differentiation of $CD103^+$ tolerogenic DCs, which in turn promote regulatory T-cell development.[27,32] In this study, we show that the stroma of the intestinal lamina propria also directly regulates T-cell responses, independent of mucosal DC regulation, indicating redundant control of T cells to limit potentially harmful inflammation in the normal intestinal mucosa. The findings presented in this study are consistent with the notion that disruption of extracellular matrix-mediated regulation of T cells in the intestinal lamina propria permits the expansion of Th1 and Th17 cells,[33] driving the inflammation that characterizes Crohn's disease pathology.

Elucidating the pathophysiology of T-cell-mediated inflammation in Crohn's disease mucosa and identification of the cytokines responsible for promoting these responses may facilitate the development of new therapeutic strategies that target these cytokines in the intestinal lamina propria of patients refractory to current therapies. Notably, our study reinforces the importance of IL-6 in promoting inflammation in Crohn's disease and indicates an important role for IL-1β in promoting T-cell proinflammatory cytokine release in the presence of TGF-β. The recent administration of a fully humanized anti-IL-1β antibody (ACZ885) in patients with rheumatoid arthritis resulted in substantial clinical improvement,[34] supporting the concept of IL-1β as a target for therapeutic intervention. Earlier studies in rabbits, mice, and humans underscored the role of IL-1β in mediating colitis.[35–38] These studies, coupled with the findings presented in this study, suggest that targeting of both local IL-1β and IL-6 may provide a more effective therapeutic strategy for patients with refractory Crohn's disease.

METHODS

Intestinal tissue and blood lymphocytes. The tissue was obtained with Institutional Review Board approval from the normal jejunum of subjects undergoing elective gastric bypass, from the normal ileum of patients undergoing colectomy for adenocarcinoma, and from the inflamed ileum of patients undergoing resection for Crohn's disease. Crohn's disease was confirmed histologically, and all donors had not received immunosuppressive therapy for 4 weeks before surgery. Blood lymphocytes were isolated from healthy donors by gradient sedimentation and purified by magnetic cell sorting using $CD4^+$ beads (Miltiney, Auburn, CA).

Stroma-conditioned media. The intestinal mucosa was dissected from the submucosa and digested to remove epithelial cells and mononuclear leukocytes as described previously.[39] The culture supernatant from cell-depleted lamina propria stroma (1 g wet weight per ml) cultured in RPMI overnight to generate S-CM[2] was sterile filtered (0.2-μm Syringe Filter; Corning, Corning, NY), tested for endotoxin and protein by enzyme-linked immunosorbent assay (ELISA) (Pierce, Rockford, IL; Bio-Rad, Hercules, CA), and stored at −70 °C. Only S-CM containing $< 1.5\,\text{Units ml}^{-1}$ endotoxin was used. Total protein concentration was determined using a bicinchoninic acid protein assay kit (Pierce). In all experiments, S-CM was used at a normalized total protein concentration of $250\,\mu\text{g ml}^{-1}$, unless otherwise indicated. S-CM derived from the normal intestinal mucosa is hereafter referred to as normal S-CM, and S-CM derived from the inflamed intestinal mucosa is hereafter referred to as Crohn's S-CM. Importantly, S-CM derived from the normal ileum and normal jejunum had similar effects on T-cell responses (data not shown).

T-cell function assays. $CD4^+$ blood T cells (10^6 per ml) obtained from normal donors were stained with carboxyfluorescein succinimidyl ester (Invitrogen, Carlsbad, CA) and incubated in RPMI with 10% HuAB serum plus IL-2 ($25\,\text{Units ml}^{-1}$; R&D Systems, Minneapolis, MN) for 1 h with (i) media, (ii) S-CM, (iii) S-CM preincubated for 30 min with or without the indicated neutralizing antibodies, (iv) S-CM plus recombinant cytokines, or (v) media with recombinant cytokines, neutralizing antibody alone, or irrelevant control antibody alone, and then stimulated with CD3/CD28 dynabeads (10^5 per ml; Invitrogen) or PHA ($5\,\mu\text{g ml}^{-1}$; Sigma-Aldrich, St Louis, MO). On day 4, supernatants were harvested for IFN-γ or IL-17 determination by ELISA (R&D Systems), and cells were stained with CD3 PEcy7, CD4 APC (BD Biosciences, San Jose, CA), or irrelevant isotype control antibody, as described previously[2] and analyzed for proliferation using an LSRII with FACS Diva software (BD Biosciences, San Jose, CA). In additional experiments, T cells were preactivated for 3 days with CD3/CD28 or PHA and then cultured for an additional 4 days with or without normal or Crohn's S-CM in the presence or absence of a second stimulation (CD3/CD28).

Cytokine determination. Among 16 normal and 15 Crohn's S-CM provided by the Human Tissue Core of the UAB Mucosal HIV and Immunobiology Center (Birmingham, AL), 7 Crohn's and 4 normal S-CM were analyzed for immunomodulating cytokines by Luminex assay (Invitrogen) (kindly performed by S.M. Wahl, NIH), according to the manufacturer's instructions. Samples for the luminex assay were randomly selected and normalized to a total protein concentration of $1.8\,\text{mg ml}^{-1}$, which was the lowest protein concentration in the assayed S-CM. On the basis of these results, which identified significantly elevated levels of IL-6 and IL-1β in Crohn's S-CM compared with normal S-CM, and our previous identification of stromal TGF-β as an important modulator,[2,4] TGF-β, IL-6, and IL-1β levels were determined by ELISA (R&D Systems) for each S-CM used in this study. Luminex and ELISA values for these cytokines were similar. IFN-γ and IL-17 in T-cell supernatants were analyzed by ELISA.

Western blot. Whole-cell preparations made from $CD4^+$ T cells (5×10^6) using standard techniques[4] were analyzed by western blot with antibodies to T-bet (1:750; Santa Cruz Biotechnologies, Santa Cruz, CA), STAT3, or p-STAT3 (1:1,000; Cell Signaling Technologies, Boston, MA), as we have described previously.[4]

Statistical analysis. Student's *t*-test was used to determine statistical significance. A paired *t*-test was used to compare experimental results

with control results, and an unpaired *t*-test was used to compare results of T cells cultured in normal S-CM to T cells cultured in Crohn's S-CM.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

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DISCLOSURE
The authors declared no conflict of interest.

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