Contribution of CD3(+)CD8(-) and CD3(+)CD8(+) T cells to TNF-alpha overexpression in crohn disease-associated perianal fistulas and induction of epithelial-mesenchymal transition in HT-29 cells

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Contribution of $CD3^+CD8^-$ and $CD3^+CD8^+$ T Cells to TNF-α Overexpression in Crohn Disease–Associated Perianal Fistulas and Induction of Epithelial-Mesenchymal Transition in HT-29 Cells

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Background: Fistulas represent a frequent and severe complication in patients with Crohn disease (CD). Tumor necrosis factor-alpha (TNF-α), transforming growth factor-beta, and interleukin (IL)-13 are known to trigger epithelial-mesenchymal transition (EMT), promoting fistula formation. Here, we investigated the role of T-lymphocytes (T cells) in fistula pathogenesis.

Methods: $CD3^+CD8^-$, $CD3^+CD8^+$, or $CD45^+CD3^-$ cells from healthy volunteers, patients with CD, and patients with CD with perianal fistula were co-cultured with HT-29 cells. The EMT, cytokine production, and mRNA expression were analyzed. Perianal CD fistula specimens were immunohistochemically stained for cytokines and their receptors. The effect of cytokines on EMT induction was investigated using an EMT spheroid model.

Results: Patients with CD with fistula revealed more $CD3^+CD8^-$ and less $CD3^+CD8^+$ T cells in blood than healthy control patients and patients with CD without fistula. In perianal fistula specimens, $CD4^+$ cells—and to a lesser extent $CD8^+$ cells—were highly present around fistula tracts. When co-cultured with HT-29 cells, both cell subsets promoted EMT-related gene expression and TNF-α production in a time-dependent manner. The $CD3^+CD8^-$ T cells from patients with CD with fistula also produced higher amounts of IL-13 than cells from healthy control patients or patients with CD without a fistula. We found that IL-22 and IL-$22R_{\alpha 1}$ were highly expressed in perianal CD fistula specimens and that IL-22 cotreatment potentiated TNF-α-induced EMT in HT-29 spheroids.

Conclusions: Our data indicate that both $CD3^+CD8^-$ and $CD3^+CD8^+$ T cells play an important role in the pathogenesis of perianal CD fistulas by the secretion of TNF-α. Our data support clinical evidence indicating that anti-TNF-α therapy is effective in fistula treatment and identify IL-13 and IL-22 as possible novel therapeutic targets for fistula therapy.

Key Words: Crohn disease, fistulas, T-lymphocytes, tumor necrosis factor-alpha

INTRODUCTION

Fistulas represent a frequent and severe complication in up to 50% of patients with Crohn disease (CD) during their disease course.[1, 2] Current knowledge about fistula pathogenesis is scarce, resulting in unsatisfactory therapeutic strategies and frequently leading to relapsing disease and a need for surgery.[3, 4] The most effective medical treatment for CD fistulas is anti-tumor necrosis factor (TNF) antibodies, but often with only limited success.[5] Interestingly, novel mesenchymal stem cell (MSC)–based therapies have had encouraging results for fistula healing with sustained fistula closure.[6-11] The first

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Author contributions: RSB designed the study; performed all experiments, data analysis, and interpretation; and wrote the first draft of the manuscript. MRS designed the study, performed fluorescence-activated cell sorting and analysis, and peripheral blood mononuclear cell (PBMC) cryopreservation and data interpretation. MCB performed cytokine measurements, PBMC cryopreservation, and data interpretation. RF, AF, and ECJ acquired human tissue specimens. AN helped with PBMC cryopreservation. SL performed parts of immunohistochemistry; LJACH, AEvdMJ, and HWV provided PBMCs and data interpretation. AR and MT: recruited patients with Crohn disease with fistula for PBMC samples. GR designed the study and obtained funding. MS conceived, designed, and supervised the study; interpreted data; revised the manuscript; and obtained funding. All authors revised and approved the manuscript.

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Conflicts of interest: The authors declare that no conflict of interest exists.

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commercial MSC product was recently approved for perianal fistula treatment in Europe.[12]

Recent analyses described an association between distinct genetic risk factors and fistula development in patients with CD.[13, 14] On a pathophysiological level, it is anticipated that epithelial-mesenchymal transition (EMT) represents the driving force for fistula development[15, 16] because EMT-associated molecules are highly expressed along fistula tracts.[15-18] Extracellular matrix remodeling enzymes, such as matrix metalloproteinases -3, -9, and -13, also seem to be involved in fistula pathogenesis.[19-22]

On a morphological level, a distinct immune cell infiltrate is detectable around CD-associated fistulas, consisting of $CD45RO^+$ T cells in the inner layer, a strong aggregation of $CD20^+$ B cells, and some $CD68^+$ macrophages in tissue surrounding the fistula.[23] Moreover, we showed that the cytokines interleukin (IL)-13 and TNF-α plus their respective receptors, along with transforming growth factor-beta, are expressed around CD-associated fistulas, supporting their pivotal role in fistula development.[15, 17, 18, 20] Interestingly, local anti-TNF-α therapy has been shown to reduce $CD4^+CD161^+$ T cell infiltrates around CD-associated perianal fistulas.[24]

Based on this knowledge, the aim of our study was to investigate the role of $CD4^+$ and $CD8^+$ T cells and typical T-cell cytokines—interferon-gamma (IFN-γ), IL-10, IL-17A, and IL-22—in the pathogenesis of CD-associated perianal fistulas.

MATERIALS AND METHODS

Ethical Considerations

Written informed consent was obtained before specimen collection, and the study was approved by the local ethics committees (Cantonal Ethics Committee of the Canton Zurich, approval no. EK-1755; Medical Ethics Committee of the Leiden University Medical Center, approval no. NL29565.000.10).

Human Patient Samples

Perianal fistula specimens of patients with 15 noninflamed fistulas (age range, 22-63 years; mean, 41 ± 11 years) and 11 patients with acutely inflamed fistulas (age range, 22-61 years; mean, 42 ± 13 years) from female and male patients with CD were collected for analysis by immunohistochemistry (IHC). Noninflamed perianal fistula samples were derived from patients with CD who were successfully treated using anti-TNF therapy. Acute inflamed perianal fistula samples were derived from patients with CD who were not responding to anti-TNF treatments. After surgical resection, tissue samples were fixed in 4% formaldehyde and embedded in paraffin. Peripheral blood mononuclear cells (PBMCs) were isolated from 30 mL whole blood of 5 healthy volunteers and, respectively, 9 or 2 patients with CD with and without perianal fistulas treated using anti-TNF-α. All patients with CD with and without fistula who donated PBMCs were selected based on having a similar treatment strategy (see Supplemental Table 1).

Isolation of PBMCs and Cryopreservation

Blood samples were processed within 30 minutes after venipuncture. Experimental details are described in the Supplemental Methods.

Fluorescence-Activated Cell Sorting

All previously isolated and cryopreserved PBMC samples were thawed and sorted for $CD3^+CD8^-$ or $CD3^+CD8^+$ (gating strategy shown in Supplemental Fig. 1) cells on the same day, using a fluorescence-activated cell sorting device, the FACS Aria III (BD Biosciences) at the University Hospital Zurich. Experimental details and antibodies are described in the Supplemental Methods.

IHC

In general, serial paraffin sections of perianal fistula samples were deparaffinized and hydrated from 100% ethanol to water before antigen retrieval was performed, followed by blocking steps, antibody staining, visualization, and dehydration/mounting. Experimental details and antibodies are described in the Supplemental Methods.

Cell Culture and Co-Culture Experiments

The human cell line HT-29 was purchased from DSMZ. The HT-29 cells were cultured as described in earlier research[20] and in detail in the Supplemental Methods. Indirect co-culture experiments were performed with sorted immune cells and HT-29 cells. Experimental details are described in the Supplemental Methods.

Three-Dimensional Multicellular Constructs as an in vitro Model for EMT

The HT-29 cells were seeded as hanging drops (spheroids) as described in earlier research.[20] Experimental details are described in the Supplemental Methods.

Quantitative Polymerase Chain Reaction

Quantitative polymerase chain reaction was performed as described in earlier research.[25] Experimental details are described in the Supplemental Methods.

Cytokine Measurements

Collected supernatant was analyzed using a U-PLEX Assay Platform (Meso Scale Diagnostics) and a commercially available duo-set ELISA (R&D Systems). Experimental details are described in the Supplemental Methods.

Statistical Analysis

Data are presented as means ± SD for a series of n experiments. Significance was determined by the Student *t* test and evaluated with GraphPad Prism 6 (GraphPad Software). We considered P values < 0.05 to be significant.

RESULTS

CD4⁺ T Cells Enriched in Patients With CD With Fistula

To investigate the role of T cells for CD fistula pathogenesis, we isolated PBMCs from healthy volunteers (HC), patients with CD without fistula (CD), or patients with CD with perianal fistula (CD-F) and sorted them for $CD3^+CD8^-$ or $CD3^+CD8^+$ cells. We detected no differences with respect to the percentage of $CD3^-$ and $CD3^+$ cells between the different groups (Supplemental Figs. 2A, B). However, PBMCs derived from CD-F revealed a significantly higher percentage of $CD8^-$ T cells indicative of $CD4^+$ T cells and a significantly lower percentage of $CD8^+$ T cells compared to CD or HC, indicating an altered T-cell composition in CD-F (Figs. 1A, B). Those data were confirmed by quantitative polymerase chain reaction analysis for *CD3D, CD4,* and *CD8A* in patient-derived PBMCs (Fig. 1C, Supplemental Figs. 2C, D). Interestingly, we detected significantly higher mRNA expression levels of the activation marker *CD69* in PBMCs from CD-F compared with PBMCs from HC (Fig. 1D).

Next, we studied staining patterns of $CD4^+$ and $CD8^+$ cells in perianal fistula tracts from CD patients by IHC. We detected strong CD4 staining around the fistula tracts, whereas staining for $CD8^+$ cells was weaker compared to CD4 cells but definitely detectable and higher than in surrounding tissue. These observations showed that the differences in the CD4:CD8 T-cell ratio observed in PBMCs from CD-F were comparable to the observations in tissue around fistula tracts (Figs. 1E, F).

Strong Expression of IL-22 in Actively Inflamed CD-Associated Perianal Fistulas

Having shown that $CD4^+$ T cells are present in higher numbers in CD-F, we further dissected the specific $CD4^+$ T-cell

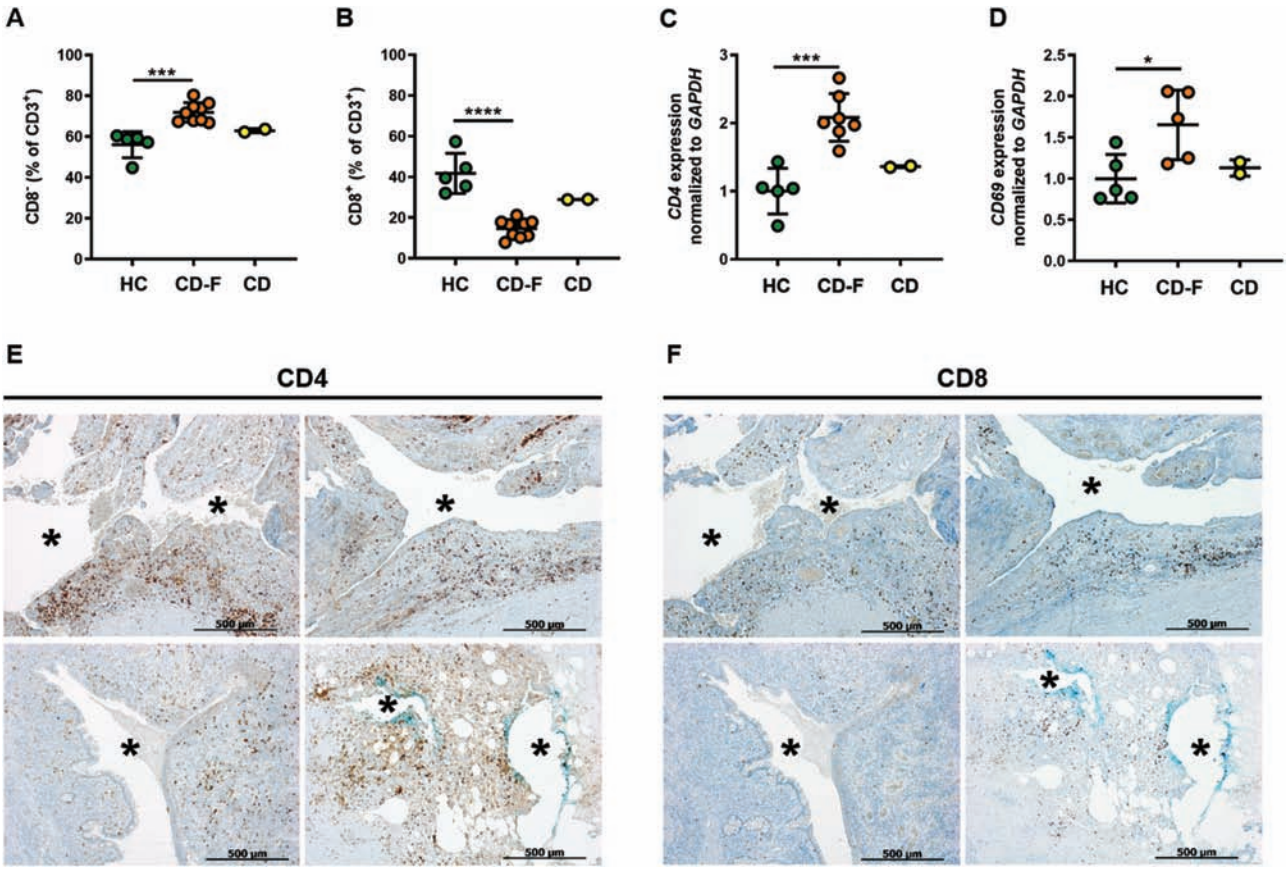

FIGURE 1. Higher $CD4^+$ T-cell expression in CD-F. PBMCs derived from HC, CD-F, and CD reveal higher proportions of $CD3^+CD8^-$ T-lymphocytes in samples from CD-F than from HC and CD (A) and fewer $CD3^+CD8^+$ T cells (B). Complementary expression patterns confirmed on mRNA gene expression level for *CD4* (C) and higher *CD69* expression (D). Quantitative polymerase chain reaction analysis was performed on the same PBMC samples as used for panels A and B before FACS, but because of varying cell numbers and low RNA yield not all samples could be included (HC, n = 5; CD-F, n = 7 for *CD4* and n = 5 for *CD69;* CD, n = 2). Similar expression ratio of CD4:CD8 T cells shown by IHC in perianal acutely inflamed fistula tissue sections from 4 different CD (E, F); represented are consecutive sections of each patient sample stained for CD4 and CD8, respectively. *$P \le 0.05$, ***$P \le 0.001$, and ****$P \le 0.0001$ for FACS quantification in panels A and B: HC (n = 5), CD-F (n = 9), and CD (n = 2). Asterisk refers to the fistula lumen; scale bars 500 µm in panels E and F. FACS indicates fluorescence-activated cell sorting.

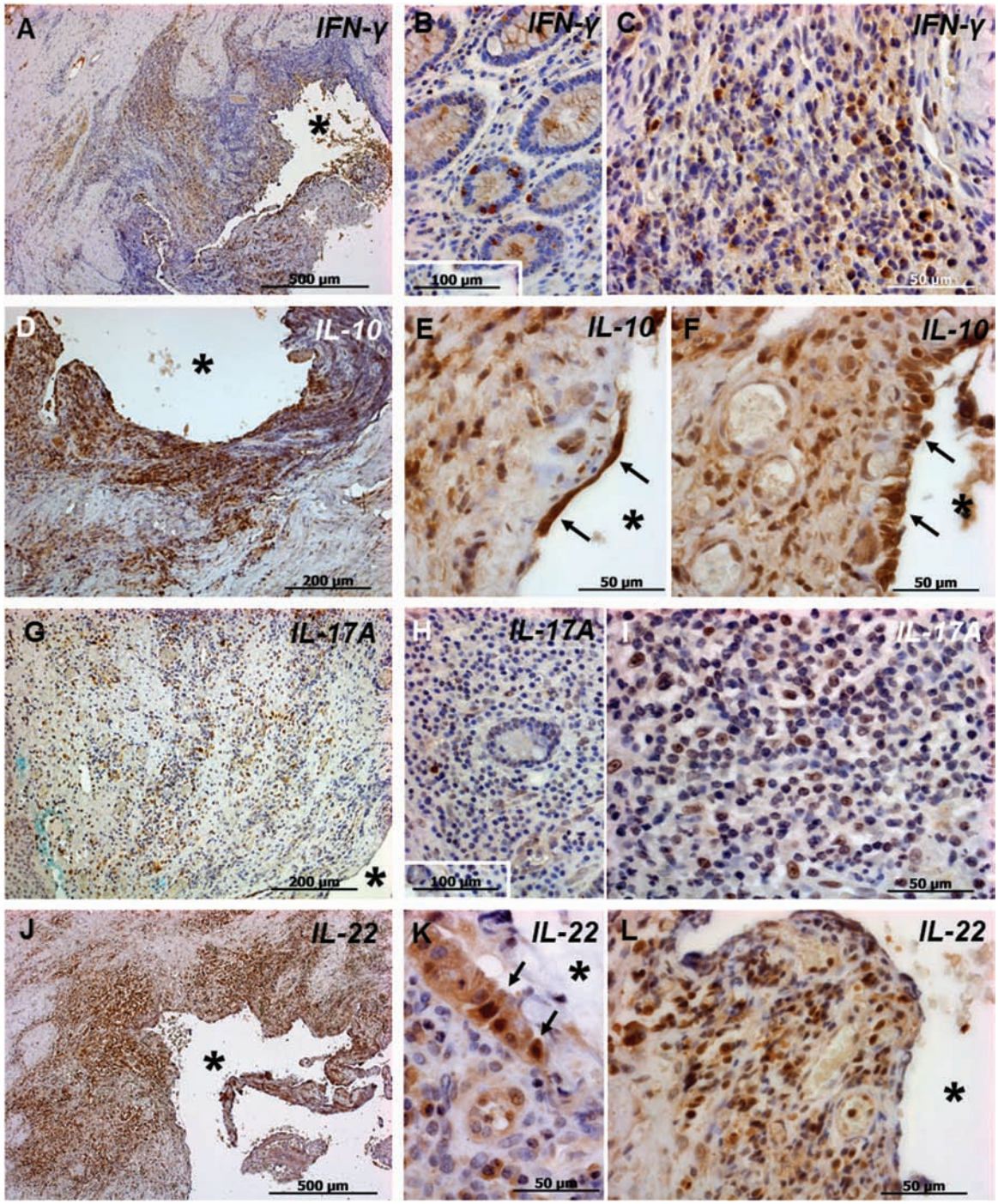

FIGURE 2. T-cell-derived cytokine expression in perianal acute inflamed fistulas from CD. Representative images of fistula tissues with IHC staining for IFN-γ reveal upregulated IFN-γ levels around the fistula tract (A, C). A particular expression pattern among the IECs of the crypts (B). IL-10 is clearly highly expressed (D–F). TCs lining the fistula tract exhibit intense staining (black arrows in E, F); likewise, cells surrounding the fistula tract are IL-10^{+} (D–F). Upregulated IL-17A production around the tract (G, I) together with expression among crypt IECs (H). Strongest expression for IL-22 (J–L). In addition to the detection in TCs (black arrows in K), IL-22 is present throughout all fistula specimens. Distinct strong expression around the fistula tract (J, L). Asterisk refers to the fistula lumen.

population to identify relevant T helper cell (Th) subsets. We found that IFN-γ, the signature cytokine of Th1 cells, showed robust expression in perianal fistula tissue. We found IFN-γ staining in the inflammatory infiltrate around the fistula tract (Figs. 2A–C) and a particular expression between the intestinal epithelial cells (IECs) of the crypts close to the tract,

likely originating from intraepithelial lymphocytes (Fig. 2B). The anti-inflammatory immunoregulatory cytokine IL-10 also revealed a strong staining intensity in acutely inflamed fistula tissue samples (Figs. 2D–F). In particular, intense IL-10$^+$ staining was visible in transitional cells (TCs; black arrows in Figs. 2E, F) and in potential lymphocytes around the fistula tract and in fibrotic areas surrounding the fistulas (Fig. 2D). The Th17 cell-derived cytokine IL-17A was overall less detectable around inflamed fistula tracts (Figs. 2G–I). However, the majority of samples still revealed several IL-17A$^+$ cells around the fistula tract and in fibrotic areas (Figs. 2G, I). We observed a similar expression pattern for IL-17A at the crypts (Fig. 2H) as described for IFN-γ (Fig. 2B), although it was less intense. However, the predominant cytokine in acutely inflamed perianal fistula samples was IL-22, which showed strong staining intensity in all analyzed samples (Figs. 2J–L). The IHC staining revealed IL-22$^+$ cells throughout the entire tissue sample. A particularly strong staining intensity was detectable along the fistula tract and in fibrotic areas (Fig. 2J), IL-22$^+$ TCs (Fig. 2K), and lymphocytes along the tract (Fig. 2L).

Distinct Cytokine Expression Pattern in Noninflamed CD-Associated Perianal Fistulas

We next studied the expression of cytokines and their receptors in noninflamed perianal CD fistula specimens. In

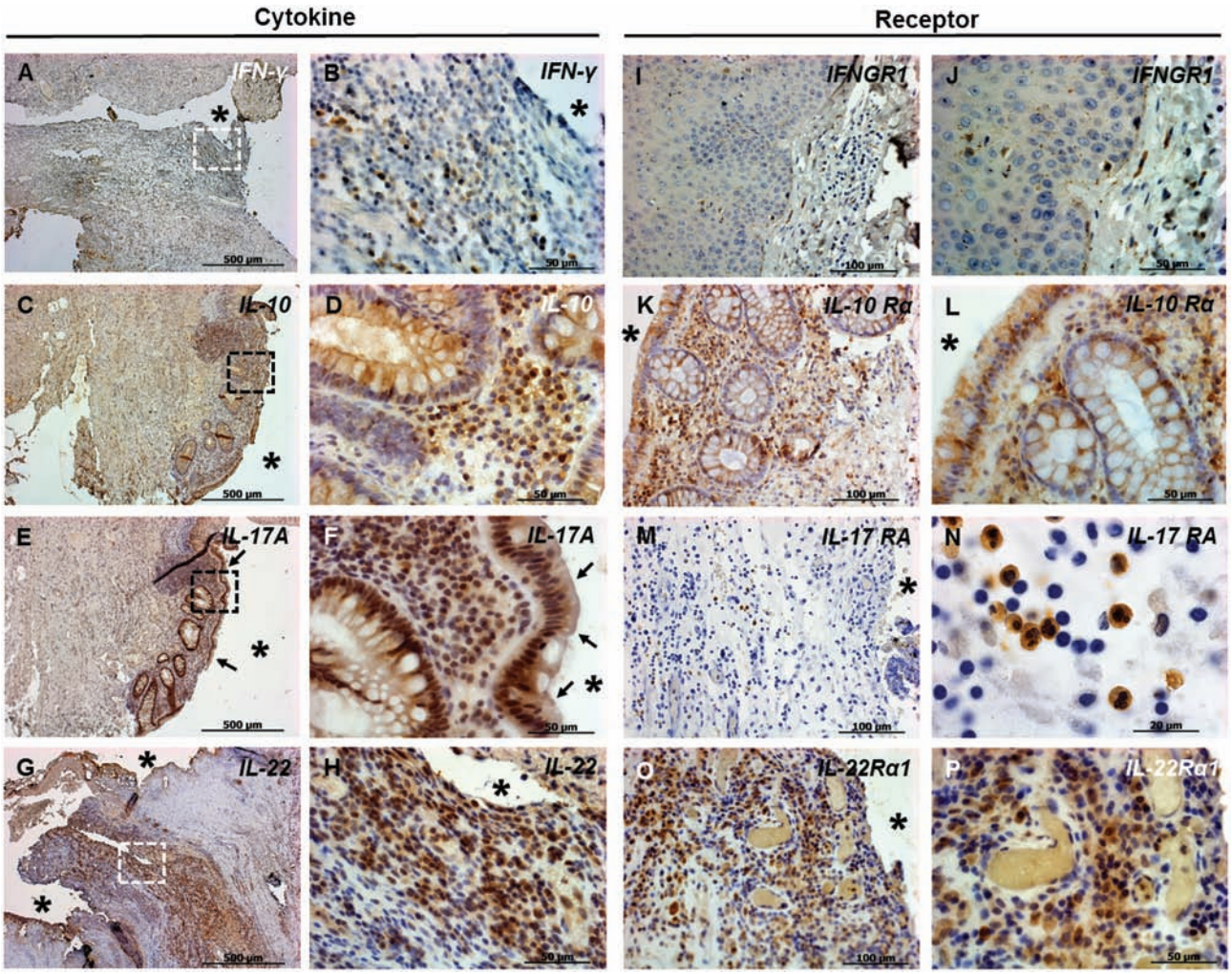

FIGURE 3. IHC analysis results showing T-cell-derived cytokine and respective receptor subunit expression in perianal noninflamed fistulas of CD. Representative images of fistula specimens show slight IFN-γ expression (A, B) but potently upregulated levels of IL-10, especially along the fistula tract (C, D). Strong expression for Th17 cell-derived cytokines IL-17A and IL-22 (E–H). Both cytokines are highly expressed at the fistula tract and in the tissue surrounding the tract. IL-17A staining also present in TCs (black arrows in E, F). Strong expression of IL-22 along and around the fistula tract (G, H). Respective inset in first column (A, C, E, G) is represented in magnification ×400 to the immediate right (B, D, F, H). IHC detected just a few IFNGR1$^+$ cells, distant from the fistula tract (I, J). IL-10 Rα revealed an expression pattern similar to that observed for IL-10 (K, L). In contrast to the high expression of IL-17A, only weak staining of the receptor IL-17 RA (M, N). Expression limited to neutrophils located in the lamina propria and submucosa (N). Strong expression of IL-22Rα1, with a staining pattern similar to that of IL-22 (O, P). Images in the third column are shown in the right-hand column in magnification ×400 or ×1000 (J, L, N, P). Asterisk refers to the fistula lumen.

contrast to IFN-γ in actively inflamed samples, we found IFN-γ to be less expressed around the fistula tract (Figs. 3A, B). Although IFN-γ-positive cells were located in the lamina propria, cells lining the fistula tract showed almost no expression (Fig. 3B). Correspondingly, we found only a limited amount of cells expressing the receptor subunit IFNGR1 (Figs. 3I, J). In addition, IFNGR1$^+$ cells were detected distant to the fistula tract.

We found that IL-10 expression was clearly detectable, especially along the fistula tracts (Figs. 3C, D), but less intensely than in acutely inflamed specimens (Figs. 2D–F). Nevertheless, we observed intense staining for IL-10 by round-shaped cells, likely lymphocytes, situated in the lamina propria along the fistula tract, below the TC/IEC layer. Among the TCs, we noticed only some IL-10$^+$ cells (Figs. 3C, D). The expression pattern of the receptor subunit IL-10 Rα was similar as that observed for the IL-10 cytokine (Figs. 3K, L).

In contrast to actively inflamed perianal fistula samples, we detected strong staining intensities for IL-17A in noninflamed perianal fistula specimens (Figs. 3E, F). Both the IECs of crypts adjacent to the tract and the TCs revealed intense staining (Figs. 3E, F). Moreover, many round lymphocyte-like cells expressed high amounts of IL-17A along the fistula tract (Figs. 3E, F) and in surrounding tissue. However, unlike the strong expression of the cytokine, its receptor, IL-17RA, was less strongly expressed—only a few IL-17RA$^+$ cells were detectable (Figs. 3M, N). We did not find IL-17RA$^+$ cells along the fistula tract but rather in the lamina propria and submucosa (Figs. 3M, N) and in fibrotic areas. Expression was limited to polymorphonuclear cells, likely representing neutrophils (Fig. 3N).

Similar to acutely inflamed specimens, IL-22 and the respective receptor subunit IL-22Rα1 were highly expressed in noninflamed fistula tissue (Figs. 3G, H, O, P). Particularly around the fistula tract we observed lymphocytes and TCs revealing strong IL-22 staining (Figs. 3G, H). Likewise, the receptor subunit IL-22Rα1 was abundantly expressed (Figs. 3O, P), and its expression pattern was similar to that of IL-22. In half of the analyzed samples, we detected even more IL-22Rα1$^+$ cells than IL-22$^+$ cells. Despite differences in the expression pattern of the investigated cytokines in actively inflamed and noninflamed perianal CD-associated fistulas, IL-22 always presented as the most abundant and intense staining in both sample types.

CD-F-Derived CD3$^+$CD8$^+$ T Cells Are Early and CD3$^+$CD8$^-$ T Cells Are Late EMT Inducers

Having shown a distinct expression pattern of T cells and T-cell-derived cytokines in CD-F, we next aimed to identify which T-cell subset contributed to the onset of EMT during fistula pathogenesis on a molecular level by performing indirect co-culture experiments. We co-cultured HT-29 cells for 24 hours and 48 hours with sorted and activated CD3$^+$CD8$^-$, CD3$^+$CD8$^+$, or CD45$^+$CD3$^-$ cells (described in Fig. 1). After 24 hours, we detected no significant differences in the mRNA expression levels of EMT-associated genes in HT-29 cells co-cultured with CD3$^+$CD8$^-$ T cells of the different patient groups (Figs. 4A–D, Supplemental Figs. 3A, B). However, CD3$^+$CD8$^-$ lymphocytes derived from CD-F induced a trend for elevated *SNAI1* and reduced *CDH1* expression as compared with HT-29 cells with lymphocytes derived from HC (Fig. 4A, B). In contrast to CD3$^+$CD8$^-$ cells derived from CD-F, CD3$^+$CD8$^+$ cells derived from CD-F induced mRNA expression levels of the EMT markers *SNAI1, ETS1, and ITGB6* and significantly reduced levels of *CDH1* after 24 hours (Figs. 4E–H) when compared with effects induced by CD3$^+$CD8$^+$ cells derived from HC, suggesting a strong impact on EMT onset. The co-culture with CD3$^-$ cells revealed no significant effects on EMT-related gene expression between all 3 groups after 24 hours (Figs. 4I–L and Supplemental Figs. 3F–H).

After 48 hours, CD3$^+$CD8$^-$ cells derived from CD-F induced elevated mRNA expression levels of *SNAI2* (Supplemental Fig. 3K) and significantly reduced levels of *CDH1* (Fig. 4N) in co-cultured HT-29 cells when compared with effects induced by CD3$^+$CD8$^-$ cells derived from HC or from CD. Notably, after 48 hours, CD3$^+$CD8$^-$ cells derived from CD increased the mRNA expression levels of *ETS1* in HT-29 cells (Fig. 4O). After 48 hours, the co-culture of CD3$^+$CD8$^+$ cells derived from CD-F with HT-29 cells resulted in increased mRNA levels of *SNAI1* (Fig. 4Q) and *DKK1* (Supplemental Fig. 3M) in HT-29 cells, compared with the effects with co-cultured CD3$^+$CD8$^+$ cells derived from HC. Similar to CD3$^+$CD8$^-$ cells, CD3$^+$CD8$^+$ cells derived from CD also enhanced mRNA levels of *ETS1* and *ITGB6* after 48 hours in comparison to the effects induced by cells derived from either CD-F or HC (Figs. 4S, T). These data suggest that CD3$^+$CD8$^+$ T cells derived from CD-F already induce EMT-related gene expression in IECs after 24 hours of co-culture. In contrast, CD3$^+$CD8$^-$ T cells from both CD and CD-F only induced EMT-related gene expression events after 48 hours. In addition, after 48 hours, CD3$^-$ cells derived from CD-F induced higher levels of the EMT-associated molecules *SNAI1* and *SNAI2* and lower levels of CDH1 (Figs. 4U, V, Supplemental Fig. 3Q). Notably, the gene expression values measured for samples exposed to CD3$^-$ cells were lower than the values measured with CD3$^+$CD8$^-$ or CD3$^+$CD8$^+$ cells, indicating a less important impact of the CD3$^-$ cells on EMT.

TNF-α as Signature Cytokine of CD3$^+$CD8$^-$ and CD3$^+$CD8$^+$ T Cells Derived From CD-F

Having confirmed that T cells from CD-F showed high efficacy in inducing EMT ex vivo and induced a gene expression pattern in HT-29 cells different than in T cells from CD, we next analyzed the supernatant of the lower co-culture compartment containing the immune cells. Here, we detected high amounts

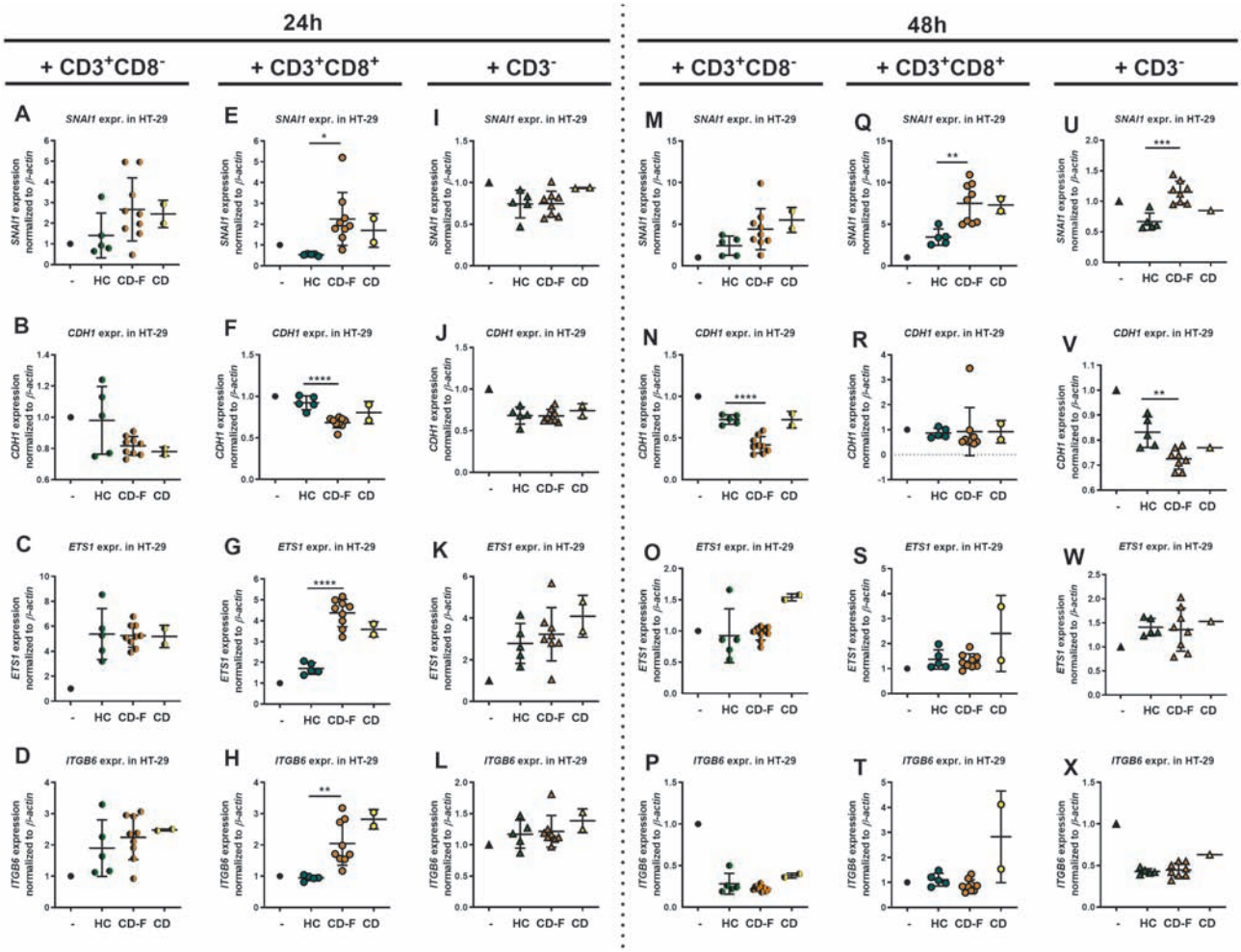

FIGURE 4. EMT-related gene expression in HT-29 monolayer co-cultured for 24 hours and 48 hours without leukocytes (-), or with sorted $CD3^+CD8^-$, $CD3^+CD8^+$ or $CD45^+CD3^-$ immune cells derived from HC, CD-F, or CD. After 24 hours of co-culture with $CD3^+CD8^-$ cells, no significant changes in mRNA expression level of *SNAI1* (A), *CDH1* (B), and *ITGB6* (D) were detectable between the 3 different groups. Only *ETS1* was upregulated compared to untreated cells (C). After 24 hours of co-culture with $CD3^+CD8^+$ cells, T cells from CD-F induced significant changes in *SNAI1* (E), *CDH1* (F), *ETS1* (G), and *ITGB6* (H) expression. 24 hours of co-culture with $CD3^-$ cells revealed no significant changes (I–L). After 48 hours of co-culture with $CD3^+CD8^-$ cells, T cells from CD-F showed a trend for higher *SNAI1* expression (M) and significantly reduced *CDH1* (N). $CD3^+CD8^-$ cells from CD induced changes for *ETS1* expression after 48 hours (O) and *ITGB6* expression did not differ (P). Co-culture of $CD3^+CD8^+$ cells for 48 hours induced enhanced *SNAI1* expression with cells from CD-F (Q), whereas *CDH1* expression showed no more differences between all groups (R). $CD3^+CD8^+$ T cells from CD triggered increased expression of *ETS1* (S) and *ITGB6* (T) after 48 hours. After 48 hours of co-culture with $CD3^-$ cells, gene expression differences were observed for *SNAI1* (U) and *CDH1* (V) but not for *ETS1* (W) or *ITGB6* (X). $^*P \le 0.05$, $^{**}P \le 0.01$, $^{***}P \le 0.001$, and $^{****}P \le 0.0001$; - (n = 1 for each condition), HC (n = 5), CD-F (n = 9 for $CD3^+CD8^-$ and $CD3^+CD8^+$ and n = 8 for $CD3^-$), and CD (n = 2 for $CD3^+CD8^-$ [24 hours, 48 hours], $CD3^+CD8^+$ [24 hours, 48 hours], and $CD3^-$ [24 hours] and n = 1 for $CD3^-$ [48 h]).

of TNF-α secreted by both $CD3^+CD8^-$ and $CD3^+CD8^+$ cells derived from CD-F after 24 hours (Figs. 5A, E) and also after 48 hours (Figs. 5C, G). Interestingly, TNF-α secretion levels by $CD3^+CD8^-$ cells derived from CD were found to be clearly lower than those of cells derived from CD-F and only $CD3^+CD8^+$ cells derived from CD-F secreted significant amounts of TNF-α, showing a striking difference from $CD3^+CD8^+$ cells derived from CD. Compared to T-cell-derived expression levels of TNF-α, $CD3^-$ cells secreted very low amounts of this cytokine and showed no significant differences between patient groups at 24 hours and 48 hours but a trend for slightly more TNF-α expression with samples derived from CD-F (Figs. 5I, J).

Notably, $CD3^+CD8^-$ cells from CD-F secreted significant amounts of IL-13 after 24 hours but not after 48 hours (Figs. 5B, D). In contrast, $CD3^+CD8^+$ cells derived from CD-F secreted significant levels of IL-13 after 24 hours and 48 hours, albeit in minor amounts compared to $CD3^+CD8^-$ cells. The $CD3^+CD8^+$ cells derived from CD showed a high expression of IL-13 after 24 hours and 48 hours (Figs. 5F, H). The $CD3^-$ cells revealed hardly any detectable IL-13 secretion

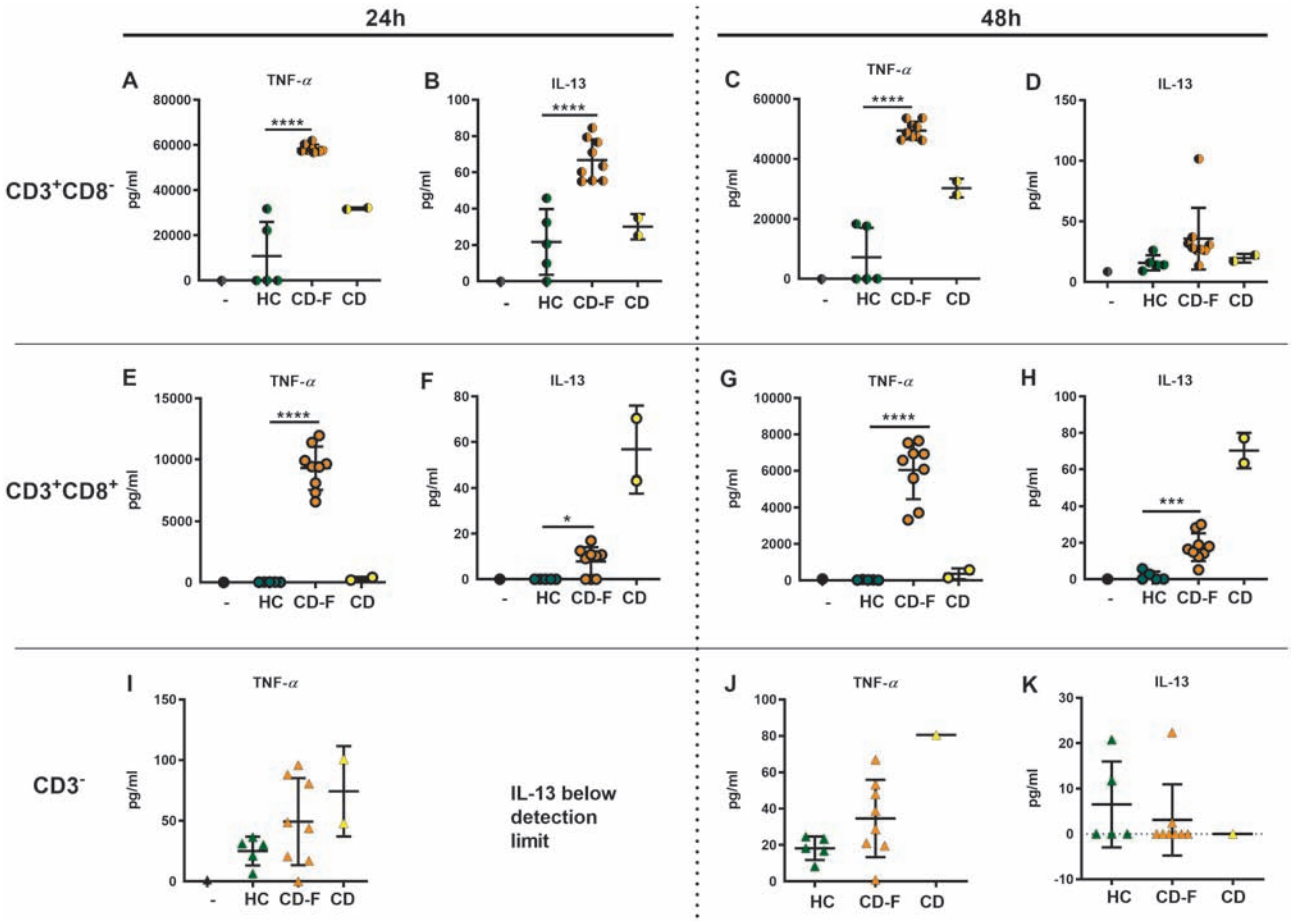

FIGURE 5. TNF-*α* and IL-13 expression levels after 24 hours and 48 hours in supernatant of the lower co-culture compartment containing no leukocytes (-) or sorted $CD3^+CD8^-$, $CD3^+CD8^+$, or $CD45^+CD3^-$ immune cells derived from HC, CD-F, or CD. $CD3^+CD8^-$ cells from CD-F significantly overexpressed TNF-*α* (A) and IL-13 (B) after 24 hours. Significant overexpression of TNF-*α* still detectable after 48 hours (C), and IL-13 expression did not differ between the 3 groups anymore (D). $CD3^+CD8^+$ cells from CD-F also with significant overexpression of TNF-*α* after 24 hours (E) and 48 hours (G). Elevated IL-13 expression detected by $CD3^+CD8^+$ cells from CD after 24 hours (F) and after 48 hours (H). $CD3^-$ cells expressed only very low levels of TNF-*α* (I, J) and IL-13 (K) in all sample groups for both 24 hours and 48 hours. *$P \leq 0.05$, ***$P \leq 0.001$, and ****$P \leq 0.0001$; - (n = 1 for each condition), HC (n = 5), CD-F (n = 9 for $CD3^+CD8^-$ and $CD3^+CD8^+$ and n = 8 for $CD3^-$), and CD (n = 2 for 24 hours, $CD3^+CD8^-$ [48 hours], and $CD3^+CD8^+$ [48 hours] and n = 1 for $CD3^-$ [48 hours]).

among all 3 groups (Fig. 5K). We found no biologically relevant differences in the secretion levels for IL-22, IFN-γ, IL-10, IL-17A, TGF-β, and IL-4 between different groups in the supernatant of $CD3^+$ cells (Supplemental Figs. 4, 5). Overall, these data support the hypothesis that TNF-α is the major driving force for fistula development in patients with CD; we show a unique TNF-α secretion of $CD3^+CD8^+$ T cells derived from CD-F.

IL-22 Inducing EMT and Cell Migration in vitro

Having shown that T cells derived from the peripheral blood of CD-F primarily but not exclusively secrete TNF-α, we next investigated the impact of the locally produced cytokines IL-22 and IFN-γ on EMT in our 3-dimensional in vitro model using HT-29 spheroids. Morphological analysis of the 3-dimensional multicellular constructs revealed an increase of separated cells and a more fringed borderline for spheroids treated with IL-22 or IFN-γ after 1 day but especially after 7 days compared with the untreated control spheroids (Fig. 6A). Disaggregation of the spheroids served as an indicator for EMT.[20] By mRNA analysis we found that IL-22 controlled the expression of genes being associated with EMT and cell invasiveness in the spheroid model after treatment for 1 day (Figs. 6B–E). We detected a significant upregulation of *ITGB6* (Fig. 6B), in accordance with Bates et al,[26] along with a significant increase of the *ETS1* transcription factor (Fig. 6C). In addition, *DKK1* expression was clearly downregulated (Fig. 6D) and the EMT marker and transcription factor *SNAI2* was not altered (Fig. 6E). At 7 days, IL-22-induced effects on gene expression were no longer detectable.

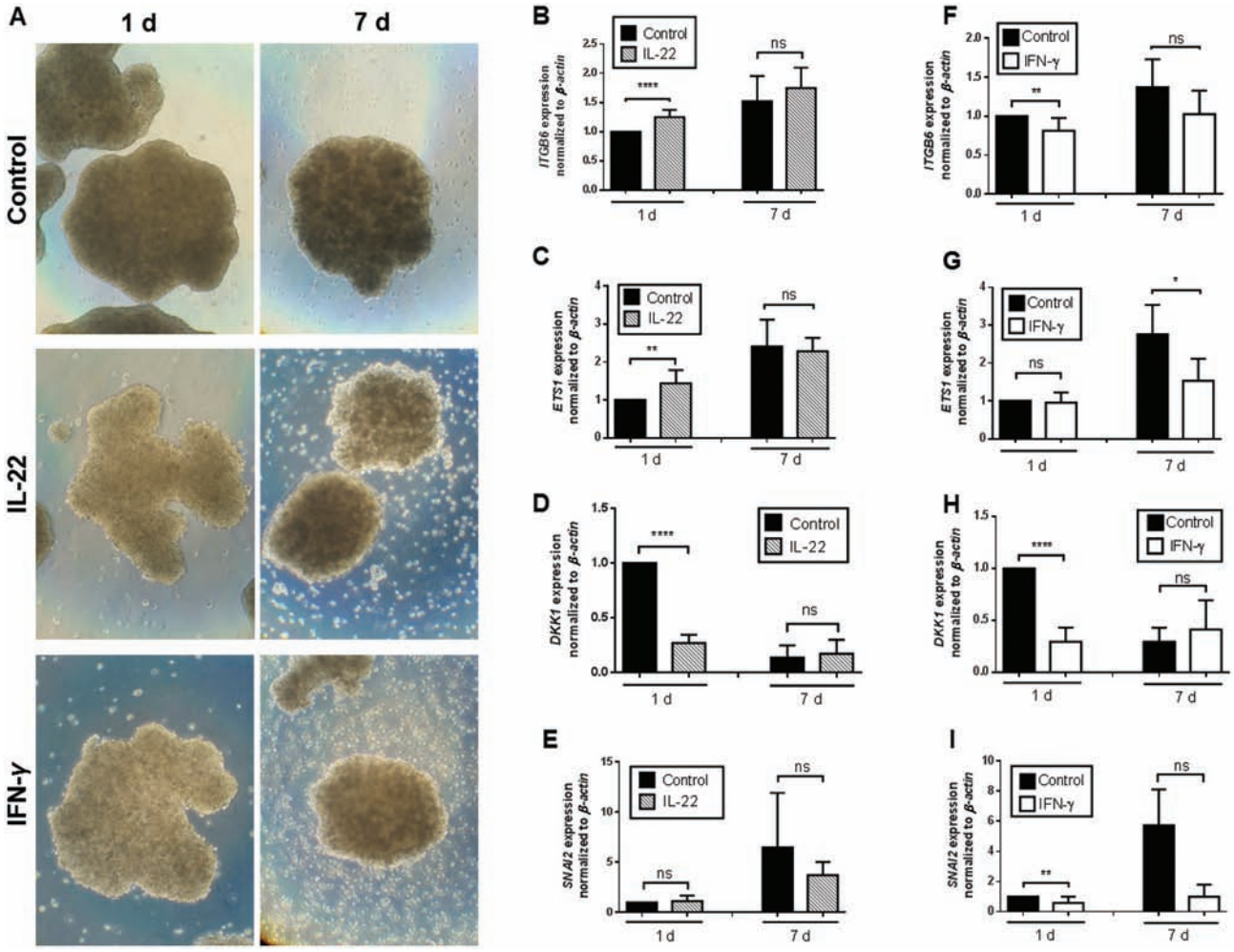

FIGURE 6. HT-29 cells grown as hanging drops either left untreated (control) or stimulated with IL-22 (100 ng/mL) or IFN-γ (100 ng/mL) for 1 or 7 days. Morphology shown by representative microscopy images (10-fold magnification) on the left; mRNA expression levels of EMT-related genes presented by bar graphs on the right. Especially after 7 days, both cytokines induced disassembly of the spheroids, indicated by the increased number of single cells in the media (A). After 1 day, IL-22 induced mRNA expression of *ITGB6* (B) and *ETS1* (C) and decreased *DKK1* levels (D), further indicating a stimulatory role on EMT. After 7 days, IL-22 stimulated spheroids showed a nonsignificant trend for downregulated *SNAI2* mRNA expression levels (E). Spheroids exposed to IFN-γ for 24 hours showed significant downregulation of *ITGB6* (F), *DKK1* (H), and *SNAI2* (I). After 7 days, *ITGB6* and *SNAI2* downregulation was still detectable although the effect was not significant anymore (F, I); *DKK1* was more expressed (H). *ETS1* was expressed significantly lower after 7 days (G). Significant differences compared to the respective controls shown by $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{****}P < 0.0001$; ns, nonsignificant; n = 5 in each group.

In contrast to IL-22, IFN-γ caused a significant reduction of *ITGB6* after 1 day and a significant reduction of *ETS1* expression after 7 days (Figs. 6F, G). Levels of *DKK1* and *SNAI2* were significantly reduced after 1 day of treatment (Figs. 6H, I). The downregulation of these genes indicated an inhibition rather than an induction of EMT by IFN-γ. Bates et al[26] reported that *ETS1* mediates an increase of *ITGB6* expression. This finding fits our observation of downregulated expression levels of *ITGB6* (Fig. 6F), further indicating a repressive role of IFN-γ on EMT. However, the significant downregulation of *DKK1* expression after 1 day (Fig. 6H) is conflicting. The cytokines IL-10 and IL-17A had no effect on EMT markers in our spheroid model (Supplemental Fig. 6).

IL-22 Potentiating TNF-α-Induced Induction of EMT

Having shown that IL-22 and IFN-γ have distinct effects on EMT induction in the HT-29 spheroid model, we next tested whether those cytokines might be able to modulate the effects of TNF-α, which is likely the key cytokine in fistula pathogenesis. The TNF-α treatment for 7 days induced spheroid disaggregation, and this effect was clearly enhanced by cotreatment with IL-22 (Fig. 7A). In contrast,

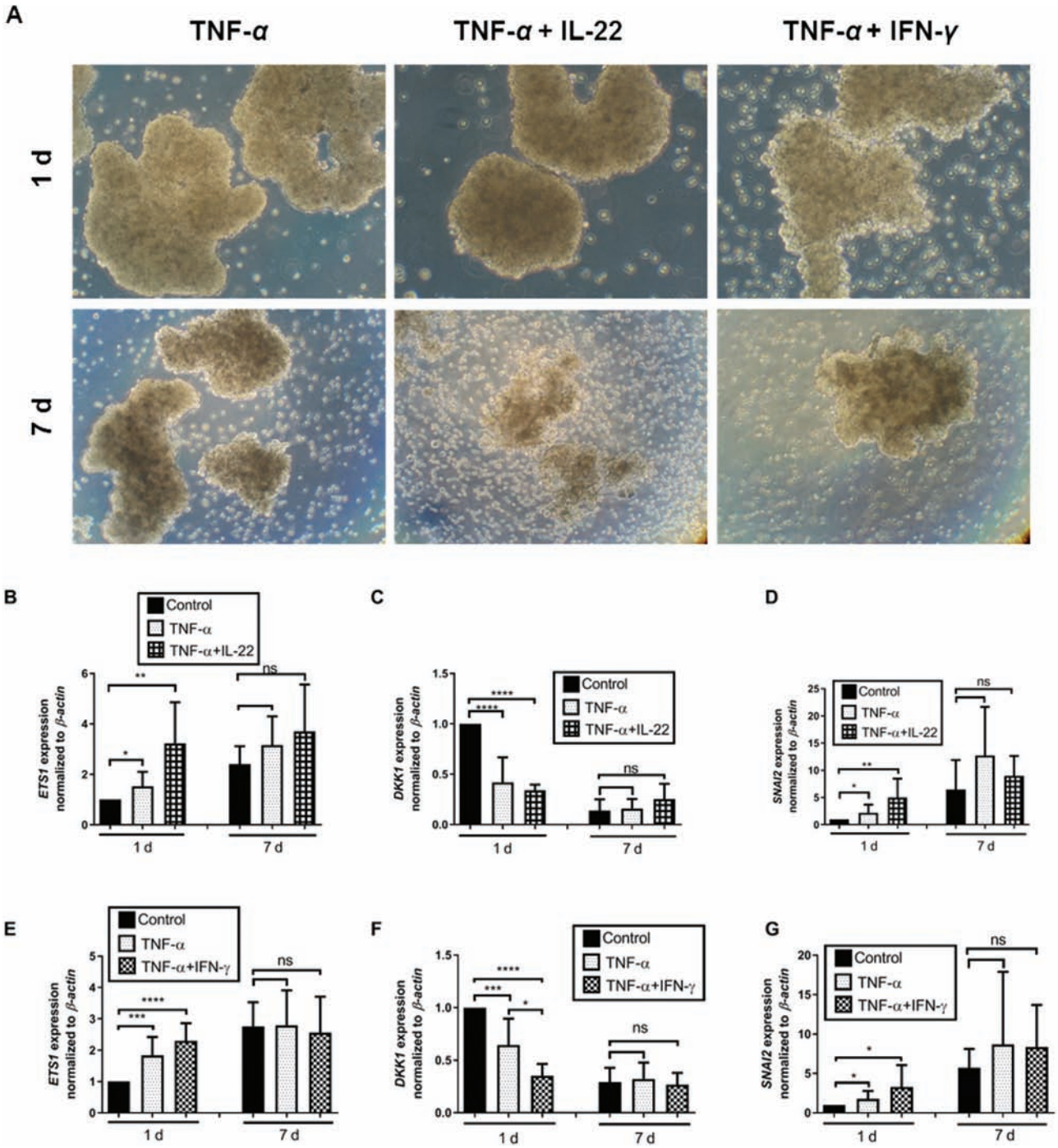

FIGURE 7. Morphology and mRNA expression levels of EMT-related genes in co-stimulated HT-29 spheroids. 3-dimensional cell constructs left untreated (control), stimulated with TNF-*α* (50 ng/mL) alone, or together with IL-22 (100 ng/mL) or IFN-γ (100 ng/mL) respectively, for 1 or 7 days. Costimulation with TNF-*α* and IL-22 presented as a potent inducer for EMT (A–D). Representative microscopy images (10-fold magnification) reveal a high amount of single cells in the media, especially after 7 days (A). Overexpression of *ETS1* (B), downregulation of *DKK1* (C), and upregulation of *SNAI2* (D) detectable after 1 day. Combined with TNF-*α*, IFN-γ revealed a stimulatory effect on EMT after 1-day treatments (A, E–G). Induced disassembly of the spheroids indicated by fringed border of cell constructs (A), also measured on mRNA expression level (E–G). Compared to the effect of TNF-*α* by itself, higher expression levels of *ETS1* (E), reduced expression of *DKK1* (F), and elevated levels of *SNAI2* (G) could be measured. After 7 days, these effects declined for both cytokines (E–G). Significant differences compared to the respective controls shown by **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001; ns, nonsignificant; n = 5 in each group.

cotreatment with neither IFN-γ nor IL-10 nor IL-17A altered the morphologic effects of TNF-α on HT-29 spheroids (Fig. 7A, Supplemental Fig. 7A). In line with the morphological appearance of the spheroids, cotreatment with IL-22 potentiated the TNF-α-induced effects on *ETS1, DKK1,* and *SNAI2* mRNA expression level, suggesting even more pronounced EMT (Figs. 7B–D). In contrast, IFN-γ-mediated inhibitory effects on EMT marker expression (reported in Fig. 6) were completely abrogated in the presence of TNF-α (Figs. 7E–G). We found that IL-10 and IL-17A had no impact on TNF-α-induced effects on EMT-related gene expression levels (Supplemental Figs. 7B–G). These data suggest that TNF-α and IL-22 may synergize in the induction of EMT and the pathogenesis of CD-associated fistulas.

DISCUSSION

In our study, we found substantial differences regarding T-cell subsets in CD-F compared to CD or HC. In particular, we detected an increase of $CD3^+CD8^-$ T cells and a decrease in $CD3^+CD8^+$ T cells in the peripheral blood of CD-F. The *CD69* gene expression showed more activity in immune cells derived from CD-F than in PBMCs derived from CD or HC. Notably, T cells from CD-F secreted high amounts of TNF-α and IL-13, supporting the importance of those cytokines for fistula pathogenesis.

These findings are in line with the hypothesis that activated T cells contribute to the development of fistula tracts in patients with CD by triggering the expression of TNF-α and IL-13. By using IHC staining for CD4 and CD8 in CD-associated perianal fistula tissue, we confirmed that the ratio of CD4:CD8 T cells in peripheral blood was comparable to the ratio detected around the fistula tract. This result prompted us to perform our experiments with PBMC-derived lymphocytes, which are easier than fistula-scraping material to collect from patients and provide higher numbers of cells. Matching our described findings here, Maggi et al[24] confirmed similar frequencies of $CD4^+$ and $CD8^+$ T cells for CD-F and showed identical $CD4^+$:$CD8^+$ expression ratios between samples derived from PBMCs and fistula curettage of the same patients. Interestingly, van Unen et al[27] described fewer $CD4^+$ T cells in the fistula tract, highlighting a role for myeloid cells. However, that study was not focused on fistulas, and functional studies with the respective cells were not performed. A study comparing lymphocyte populations derived from intestinal biopsy specimens from HC, patients with CD, and patients with ulcerative colitis found significantly higher and lower percentages of $CD3^+CD4^+$ and $CD3^+CD8^+$ cells, respectively, in CD compared with HC. Notably, those authors suggested associations between higher baseline expression levels of $CD4^+$ cells and Tregs at disease onset in patients with CD and future complications such as stricturing or penetrating disease,[28] matching our current findings.

We also showed differences in the protein expression of IFN-γ, IL-10, IL-17A, and IL-22 and their respective activating receptors between inflamed and noninflamed perianal fistulas. To characterize further the functional relevance of those cytokines for fistula pathogenesis, we performed co-culture experiments using sorted immune cells derived from the peripheral blood of HC, CD, and CD-F and HT-29 cells. We found that only T cells derived from CD-F triggered EMT-like responses in HT-29 cells, in line with the observed cytokine secretion data. Notably, IL-22 revealed synergistic effects together with TNF-α on the mRNA expression levels of genes related to EMT and cell invasiveness in the spheroid model, suggesting a potential role for IL-22 in fistula pathogenesis.

By analyzing the supernatant from the indirect co-culture experiments, we identified TNF-α as a key signaling molecule. Both $CD3^+CD8^-$ and $CD3^+CD8^+$ cells derived from CD-F secreted significant levels of TNF-α at 24 hours and 48 hours, although $CD3^+CD8^-$-derived levels were approximately 6- to 8-fold higher. These observations support the hypothesis of TNF-α-secreting T cells as the driving force for fistula development in patients with CD. In contrast, the minor TNF-α secretion levels determined for $CD3^-$ cells, revealing no differences between the different sample groups, suggest that those cells do not play a prominent role in fistula development. Previous research has already described TNF-α as a potent EMT-inducer contributing to fistula pathogenesis in patients with CD.[17] Accordingly, TNF-α-targeting antibodies are well-established therapies in perianal and fistulizing CD. However, T cells as the likely major source of this cytokine in CD-F are not yet described. In addition, previous research reported an important role for IL-13 in fistula formation by triggering the expression of molecules mediating cell invasiveness in TCs.[20] Those previous data are well in line with our current findings that $CD3^+$ cells derived from CD-F secreted significant amounts of IL-13 that might induce the upregulation of *SNAI2* mRNA expression in our co-cultured HT-29 cells. Overall, the findings further support the notion that IL-13 may be a useful target for fistula therapy, perhaps in addition to rather than instead of anti-TNF-α treatment in patients with CD.

We are aware that the low number of patient samples represents a limitation in our study, especially because the spread within CD-derived samples is sometimes wide. Follow-up-studies of the patients with CD who did not have a fistula at the time of sample acquisition for this study would be interesting, to investigate whether those patients are more likely to develop perianal disease. However, comparing only samples derived from CD-F with those derived from HC still revealed clear differences and distinct clustering between both sample groups. The fact that blood samples from CD-F were collected from both Swiss and Dutch patients at 2 different facilities further strengthens the reported findings that these are indeed particular characteristics for CD-F.

CONCLUSIONS

We report a pathogenetic role for differently expressed T-cell subsets in patients with CD with perianal fistulas. Our data show $CD3^+CD8^-$ and $CD3^+CD8^+$ cells as major sources of TNF-α overexpression and as triggers for EMT in HT-29 cells and suggest that targeting of IL-13 and IL-22 (locally) may provide novel approaches for fistula therapy.

SUPPLEMENTARY DATA

Supplementary data are available at *Inflammatory Bowel Diseases* online.

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