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ARTICLE

Frequencies of circulating regulatory TIGIT$^+$CD38$^+$ effector T cells correlate with the course of inflammatory bowel disease

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Disease heterogeneity hampers achieving long-term disease remission in inflammatory bowel disease (IBD). Monitoring ongoing tissue-localized regulatory and inflammatory T-cell responses in peripheral blood would empower disease classification. We determined whether regulatory and inflammatory phenotypes of circulating CD38$^+$ effector (CD62L^{neg}CD4$^+$) T cells, a population enriched for cells with mucosal antigen specificity, classify disease course in pediatric IBD patients. In healthy individuals, circulating CD38$^+$ effector T cells had a predominant regulatory component with lower frequencies of IFNγ-secreting T cells, higher frequencies of IL-10-secreting T cells and higher frequencies of inhibitory molecule T-cell immunoglobulin and ITIM domain$^+$ (TIGIT) cells than CD38^{neg} effector T cells. TIGIT expression was stable upon stimulation and marked CD38$^+$ T cells with inhibitory properties. In IBD patients with active intestinal inflammation this predominant regulatory component was lost: circulating CD38$^+$ effector T cells had increased activated CD25$^+$CD45RA^{neg} and decreased TIGIT$^+$ cell frequencies. TIGIT percentages below 25% before treatment associated with shorter duration of clinical remission. In conclusion, phenotypic changes in circulating CD38$^+$ effector T cells, in particular the frequency of TIGIT$^+$ cells, classify pediatric IBD patients and predict severity of disease course. These findings have relevance for IBD and can be exploited in graft-versus-host-disease and checkpoint inhibitor-induced inflammation in cancer.

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INTRODUCTION

Being continuously exposed to a large variety of foreign antigens, the intestinal immune system tailors its response to mount balanced inflammatory responses against pathogenic antigens and regulatory responses to harmless antigens thus avoiding excessive tissue damage. Such balancing of T-cell responses is lost in inflammatory bowel disease (IBD) patients who suffer from chronic inflammatory immune responses to commensal microbial antigens characterized by infiltration of interferon gamma (IFNγ)- and interleukin-17 (IL-17)-secreting effector T cells in the intestinal mucosa.[1] The disease is heterogeneous in terms of severity, disease course, and anatomical location. As a result, current therapeutic strategies which aim at suppressing these inflammatory effector memory T-cell responses have variable therapeutic effect. Monitoring loss of balance between inflammatory and regulatory tissue-localized T-cell responses in pediatric IBD patients is highly desired to classify patients and predict their disease course but is difficult as endoscopy is too invasive to routinely be used. Moreover, T cells isolated from the intestinal mucosa cannot be used for accurate quantification and enumeration of cell populations as biopsies are not representative of the entire mucosal surface. Instead, peripheral blood is accessible for enumeration of inflammatory versus regulatory T-cell populations and would enable longitudinal follow up. However, until now, monitoring the phenotype of the total CD4$^+$ T-cell population in peripheral blood has not yielded consistent changes in inflammatory and regulatory populations in IBD. For example, reduced frequencies of circulating CD4$^+$ T cells expressing forkhead box P3 (FoxP3) have been reported in some IBD studies[2–5] but not in others.[6,7] Monitoring frequencies of cells in the total circulating CD4$^+$ T-cell population may simply not be sensitive enough to reliably detect transient changes in inflammatory or regulatory intestinal T-cell responses.

Recently, we demonstrated that CD38 expression on peripheral blood CD4$^+$ effector (CD4$^+$CD62L^{neg}) T cells enriches for T cells with specificity for mucosal antigens.[8] Circulating CD38$^+$ effector T cells, comprising 4–10% of the total CD4$^+$ T-cell pool, are enriched in cells expressing the gut-homing chemokine receptor C–C chemokine receptor type 9 (CCR9) and β7-integrin compared to CD38^{neg} effector T cells. Conversely, cells expressing the skin-homing receptor cutaneous leukocyte-associated antigen are almost absent in the CD38$^+$ effector T-cell population but enriched in the CD38^{neg} effector T-cell population.[8] After oral gluten challenge all gluten-specific CD4$^+$ T cells in peripheral blood of celiac disease patients have the CD38$^+$ effector phenotype demonstrating specificity for intestinal luminal antigen

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is contained within this population.[8] In intestinal tissue virtually all $CD4^+$ T cells have the $CD62L^{neg}CD38^+$ phenotype.[8–10] Crucially, imprinting of CD38 expression on differentiating T cells occurs irrespectively of the inflammatory or regulatory nature of the differentiating T cell.[8]

Having established that the circulating $CD38^+$ effector T-cell population is enriched in cells with mucosal antigen specificity, we hypothesized that the composition of inflammatory versus regulatory cells in the circulating $CD38^+$ effector T-cell population detects ongoing disease activity and predicts disease course and therapy response in pediatric IBD.

RESULTS

Increased frequencies of activated T cells in the circulating $CD38^+$ effector T-cell pool during active IBD

We have recently established that percentages of peripheral blood $CD38^+$ effector ($CD4^+CD62L^{neg}$) T cells do not differ between patients with chronic intestinal disease and controls.[8] We therefore hypothesize that the composition of inflammatory versus regulatory cells within this circulating $CD38^+$ effector T-cell population detects active inflammatory disease in pediatric IBD. Thereto, we examined changes in T-cell activation in circulating $CD38^+$ effector T cells in pediatric IBD patients at time of disease diagnosis when patients have active biopsy-proven intestinal inflammation and are not yet receiving treatment, and during clinical remission while treated (Figure S1A). During active disease ("inflammation") but not during clinical remission, pediatric IBD patients exhibited increased frequencies of antigen-experienced $CD45RA^{neg}$ cells in the $CD38^+$ effector T-cell population when compared to age-matched healthy controls (Fig. 1a). Upon subdividing IBD patients in CD and UC, the increased frequencies of $CD45RA^{neg}$ cells were most prominent in CD (Figure S2A). These changes were not detectable in the total $CD4^+$ T-cell population (Fig. 1a). To detect cellular activation, we determined frequencies of cells expressing the IL-2 receptor α chain (CD25), that is induced on naive T cells after activation via T-cell receptor signaling. Frequencies of activated $CD25^+FoxP3^{neg}$ T cells were strongly increased in the $CD38^+$ effector T-cell population, but much less in the total $CD4^+$ T-cell population of pediatric IBD patients with active disease compared to controls (Fig. 1b). Patients in clinical remission did not exhibit this increased frequency of $CD25^+FoxP3^{neg}$ $^{CD38+}$ effector T cells. The frequency of $CD25^+$ cells in the $CD38^+$ effector T-cell population revealed a marked heterogeneity in patients with active disease (Fig. 1b). Increased frequencies of activated $CD25^+FoxP3^{neg}$ T cells amongst $CD38^+$ effector T cells were observed in both CD and UC patients (Figure S2B). Of note, there were no differences in white blood cell (WBC) counts or percentage of $CD38^+$ effector T cells expressing gut-homing receptors CCR9 and α4β7 between patient groups (Figure S1B and S3). Medication use is summarized in Figure S1C and gating strategy is shown in Figure S1D. Upon scheduled treatment follow up (median treatment duration 223 days) and despite a wide range of immunosuppressive treatments (Figure S1F), frequencies of $CD25^+$ cells decreased in the $CD38^+$ effector T-cell pool, but not total $CD4^+$ T cells, of all patients achieving clinical remission (Fig. 1c). These data demonstrate that monitoring activated $CD45RA^{neg}$ and $CD25^+$ T cells in the circulating $CD38^+$ effector T-cell population detects active intestinal disease in pediatric IBD while this cannot be achieved by monitoring all $CD4^+$ T cells.

As in IBD patients the balance between activated inflammatory T-cell responses and regulatory responses is lost, we next sought the reduced regulatory component that would reflect inflammatory disease. Thereto, we assessed the frequencies of circulating $FoxP3^+$ cells in the $CD38^+$ effector T-cell population in peripheral blood of IBD patients and controls. However, we detected no differences in the frequencies of $FoxP3^+$ cells in total $CD4^+$ T cells or $CD38^+$ effector T cells between patients and controls (Fig. 1d). In humans, $FoxP3^+$ T cells are functionally heterogeneous with regulatory T cells (Tregs) and a subpopulation of activated effector $CD4^+$ T cells expressing FoxP3.[11] To further investigate FoxP3 expression in regulatory versus activated $CD4^+$ T cells, we combined analysis of FoxP3 and CD45RA to distinguish naïve Tregs ($CD45RA^+FoxP3^{int}$), activated Tregs ($CD45RA^{neg}FoxP3^{hi}$) and activated effector T cells ($CD45RA^{neg}FoxP3^{int}$, as depicted in the gating strategy in Figure S4A).[12] Frequencies of activated effector T cells were increased in both cohorts of IBD patients compared to controls, whereas no differences were found in the percentage of naïve and activated Tregs between IBD patients with active disease and controls (Figure S4B). In addition, no differences were found in the frequency of $CD4^+CD25^{hi}CD127^{neg}FoxP3^+$ T cells between IBD patients and controls (Figure S4C and S4D).

Overall, these data demonstrate that enrichment for $CD38^+$ effector T cells instead of total $CD4^+$ T cells in peripheral blood detects increased frequencies of activated T cells in IBD patients with intestinal inflammation. This increased frequency normalizes during disease remission. However, frequencies of potentially regulatory populations in the $CD38^+$ effector T-cell population are difficult to monitor.

Circulating $CD38^+$ effector T cells of adult healthy controls have a predominant regulatory component with decreased inflammatory cytokine production and increased IL-10 production

We next aimed to identify the main regulatory component in the circulating $CD38^+$ effector T-cell pool. Previously, preliminary data suggested that $CD38^+$ effector T cells from adult healthy individuals are enriched in IL-10 mRNA expression when compared to $CD38^{neg}$ effector T cells homing to other sites of the body.[8] This appears in line with the preferential tolerogenic response to harmless exogenous antigens at the mucosal surface. To corroborate enrichment in regulation, purified $CD38^{neg}$ and $CD38^+$ effector T cells from adult healthy individuals were cultured with allogeneic monocyte-derived dendritic cells (DCs) in a mixed lymphocyte reaction (MLR). $CD38^+$ effector T cells from healthy individuals contained lower frequencies of IFNγ- and IL-17-secreting T cells, and higher frequencies of IL-10-secreting T cells when compared to $CD38^{neg}$ effector T cells (Fig. 2a, b). Both the frequencies and mean fluorescence intensity of HLA-DR and CD25 did not differ between cultured $CD38^{neg}$ and $CD38^+$ effector T cells (Figure S5A and S5B), excluding the possibility that the different cytokine profiles were due to overall differences in T-cell activation. Using a second method of activation, i.e. anti-CD3/CD28 stimulation, $CD38^+$ effector T cells also produced lower levels of IFNγ and IL-17 compared to $CD38^{neg}$ effector T cells (Fig. 2c). Overall, frequencies of $CD45RO^+$ (memory) or $CD45RA^+$ (naïve) cells (Figure S5C), $FoxP3^+CD25^{hi}$ (Figure S5D) and $FoxP3^+CD127^{neg}$ T cells (data not shown) did not differ between $CD38^{neg}$ and $CD38^+$ effector T-cell populations. In addition, $CD38^{neg}$ and $CD38^+$ effector T-cell populations had comparable frequencies of $Helios^+$ cells in $FoxP3^+$ Tregs frequencies suggesting similar frequencies of natural Tregs (Figure S5D).

Taken together, in adult healthy individuals, circulating $CD38^+$ effector T cells which are enriched in cells with mucosal antigen specificity, have a stronger immunoregulatory component associated with decreased inflammatory cytokine production and increased IL-10 production when compared to $CD38^{neg}$ effector T cells homing to sites such as the skin.

Healthy control $CD38^+$ effector T cells are enriched for transcripts associated with immune regulation and gut homing when compared to $CD38^{neg}$ effector T cells

Next, we set out to define cellular markers to identify this regulatory subpopulation of $CD38^+$ effector T cells. Thereto, we performed transcriptome analysis of $CD38^{neg}$ and $CD38^+$effector T cells of adult healthy individuals ($n = 3$) to find differentially expressed proteins reflecting regulatory function of $CD38^+$ effector T cells. Overall, a

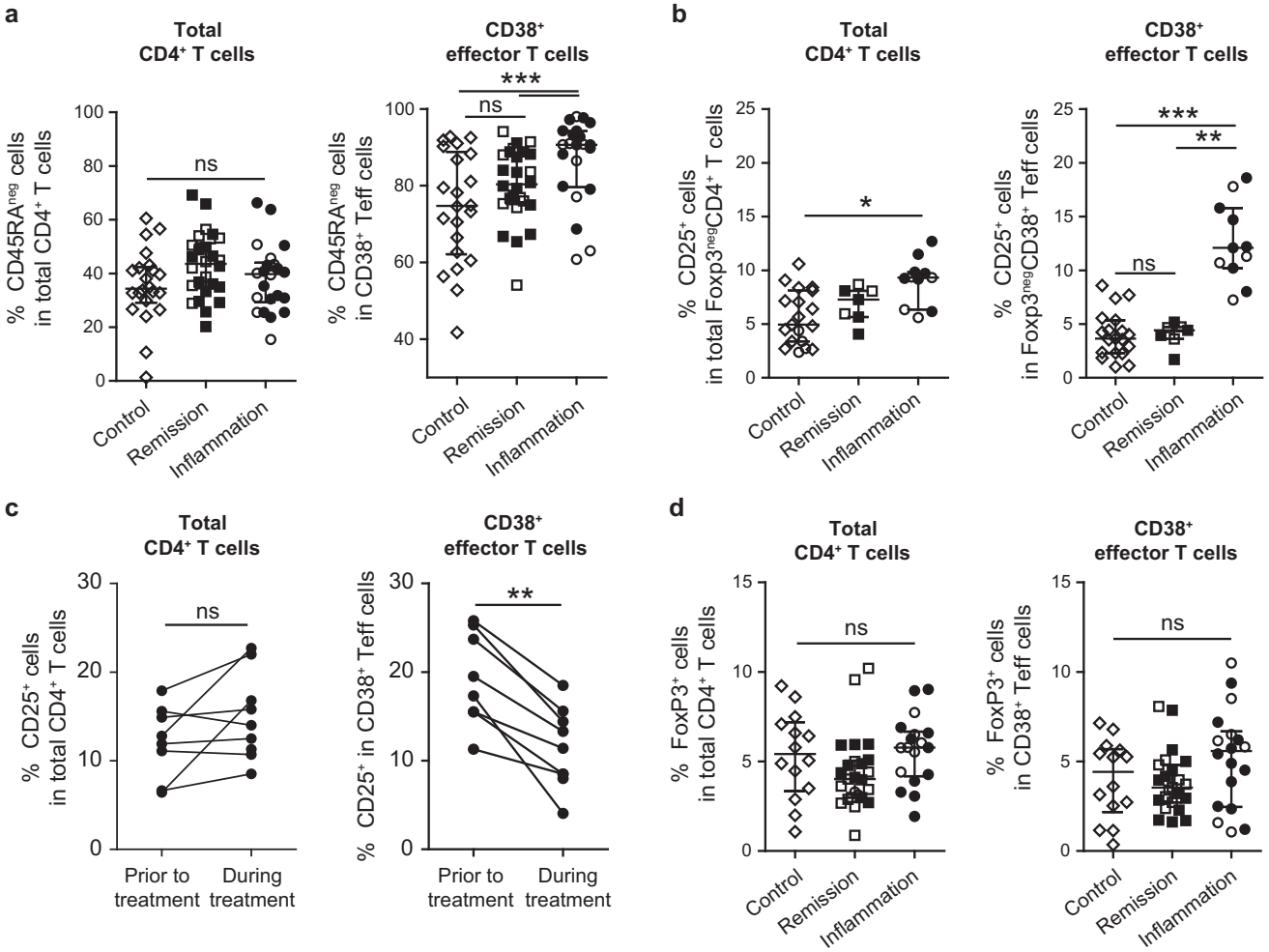


Fig. 1 Increased frequencies of activated T cells in the circulating effector CD38$^+$ T-cell pool during active IBD. Flow cytometric analysis for CD3, CD4, CD62L, CD38, CD45RA, CD25, and FoxP3 was performed on peripheral blood from pediatric IBD patients (cohort I) with biopsy-proven intestinal inflammation at diagnostic endoscopy and prior to treatment (denoted as "inflammation"), pediatric IBD patients in clinical remission during treatment (denoted as "remission") and age-matched healthy controls. Open and closed symbols depict UC and CD patients respectively. Cells were gated on single cells, CD3$^+$, CD4$^+$, and CD62L^{neg}CD38$^+$ cells (denoted as CD38$^+$ effector T cells). **a** Frequency of CD45RA^{neg} cells in the total CD4$^+$ T-cell population and CD38$^+$ effector T cells within the CD4 gate (cohort I; age-matched healthy controls, $n = 22$, IBD patients with intestinal inflammation, $n = 22$, IBD patients in remission, $n = 26$). **b** Frequency of CD25$^+$ cells gated on FoxP3^{neg} cells in the total CD4$^+$ T-cell population and CD38$^+$ effector T cells within the CD4 gate (cohort I; age-matched healthy controls, $n = 19$, IBD patients with intestinal inflammation, $n = 11$, IBD patients in remission, $n = 7$; CD25 analysis was included in a second phase of the study). **c** Frequency of CD25$^+$ cells in the total CD4$^+$ T-cell pool and CD38$^+$ effector T-cell population in peripheral blood of pediatric IBD patients at disease diagnosis (prior to treatment) and during immunosuppressive treatment (follow up in $n = 9$ patients from cohort II consisting of a total of $n = 18$; median treatment duration 223 days, IQ range 169–309). p values were calculated using a Wilcoxon signed rank test. **d** Frequency of FoxP3$^+$ cells in the total CD4$^+$ T-cell population and CD38$^+$ effector T cells within the CD4 gate. Data are expressed as median ± interquartile (IQ) range. p values were calculated using a Kruskal–Wallis H analysis followed by the Dunn's Multiple Comparison Test. Teff, effector T cell; NS, not significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

total of 145 and 167 genes were significantly over- and under-expressed in CD38$^+$ effector T cells relative to their CD38^{neg} counterparts (with an adjusted p value < 0.05; Fig. 3a). Hierarchical clustering performed with this gene set clearly distinguished CD38^{neg} and CD38$^+$ effector T cells (Fig. 3b). As expected, expression of genes encoding the gut-homing chemokine receptors *CCR9*, *CXCR6* and *CX3CR1*, as well as integrin α4 *(ITGA4)* were up-regulated in CD38$^+$ effector T cells compared to CD38^{neg} effector T cells (Fig. 3c). In addition, CD38$^+$ effector T cells had increased expression of a number of genes encoding coinhibitory receptors, such as T-cell immunoglobulin and ITIM domain *(TIGIT)*, cytotoxic T lymphocyte antigen-4 (*CTLA-4)*, Fc receptor-like protein 3 *(FCRL3)*, Hepatitis A virus cellular receptor 2 (*HAVCR2*, encoding T cell Immunoglobulin and Mucin 3; *TIM-3*) and the costimulatory molecule inducible T-cell costimulator *(ICOS*, Fig. 3c). CD38$^+$ effector T cells also showed increased expression of the gene encoding the regulatory cytokine IL-10, although significance was lost after correction for multiple testing (p value = 0.003, adjusted p value = 0.09). Based on the RNA-seq data, we selected immune regulatory genes that were enriched in the CD38$^+$ effector T-cell population and validated their differential expression with qRT-PCR analysis (Fig. 3d). Overall, these analyses provide us with candidate molecules TIGIT, CTLA-4, ICOS and TIM-3 as putative markers to monitor the IL-10-producing regulatory subpopulation within the CD38$^+$ effector T-cell pool.

The inhibitory receptor TIGIT is strongly enriched in the circulating healthy control CD38$^+$ effector T-cell pool and its expression is maintained upon cellular activation

We next analyzed whether this distinctive mRNA profile translated into cellular protein expression of TIGIT, CTLA-4, ICOS, and TIM-3 in

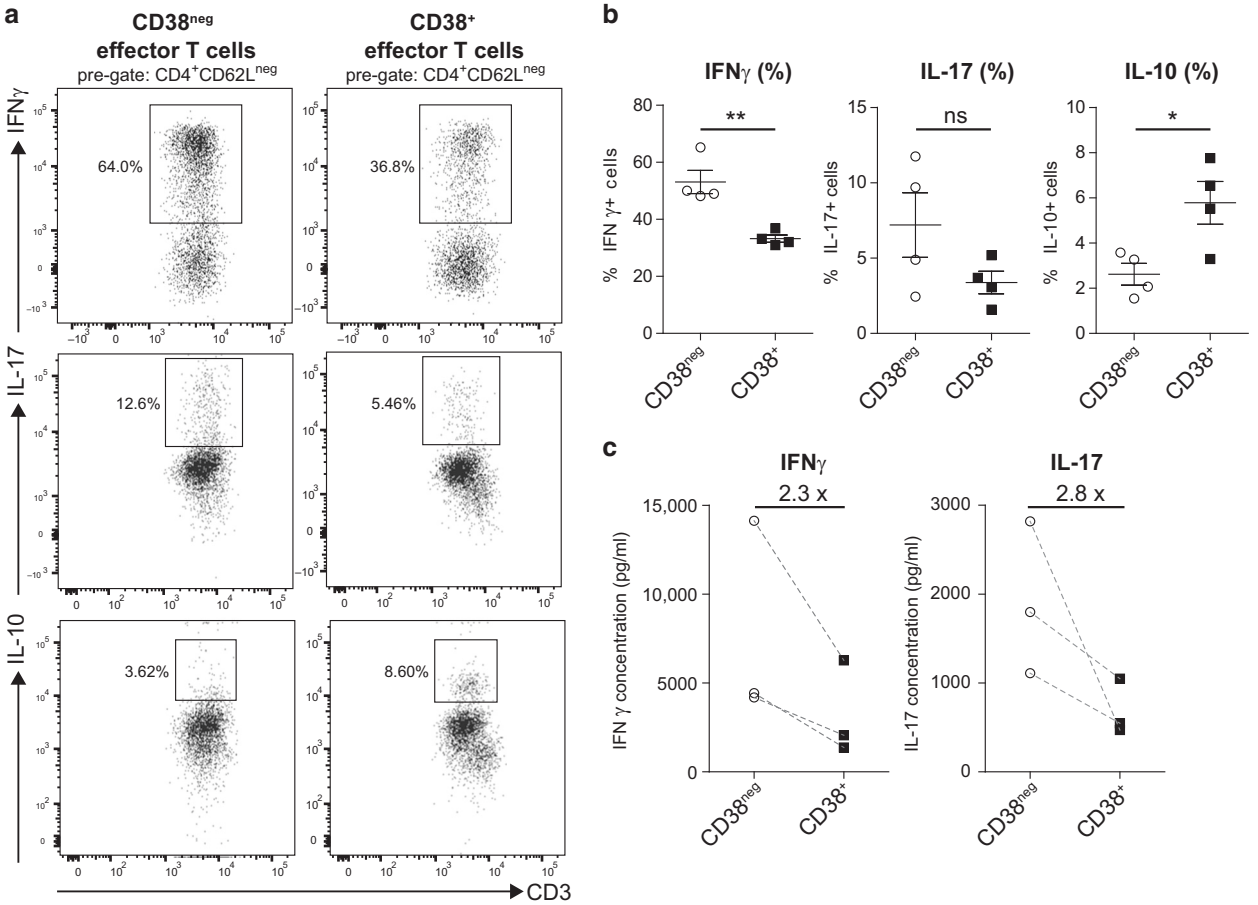


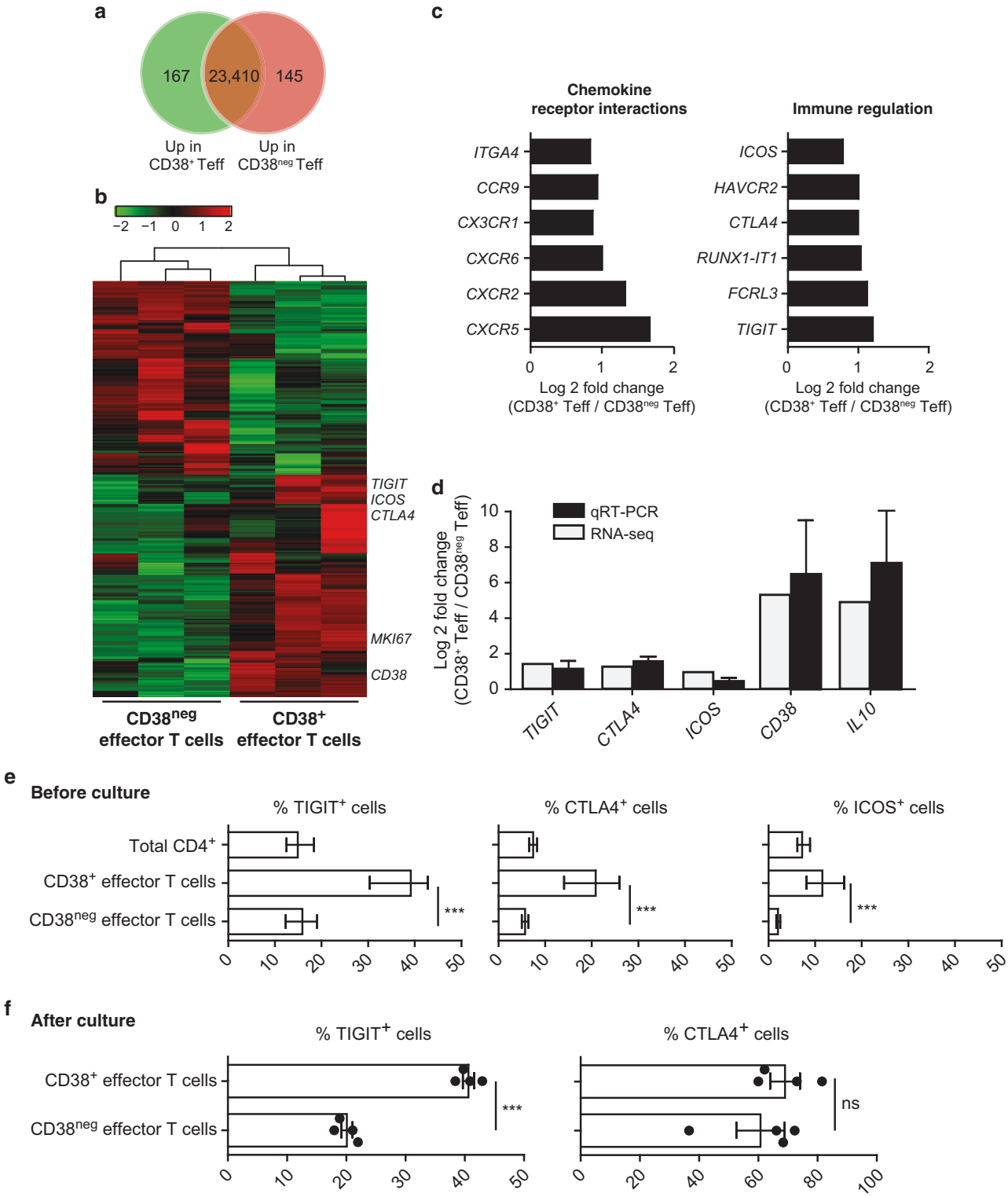
Fig. 2 Circulating CD38$^+$ T cells of adult healthy controls have a predominant regulatory component with decreased inflammatory cytokine production and increased IL-10 production. **a**, **b** Purified adult healthy control CD38^{neg} and CD38$^+$ effector T cells were cultured for 72 h with mature monocyte-derived DCs (ratio 10:1). **a** Representative dot-plots of the percentage of IFNγ, IL-17 and IL-10 positive cells in CD38^{neg} and CD38$^+$ effector T cells at 72 h are shown. **b** Percentages of IFNγ, IL-17 and IL-10 positive cells in CD38^{neg} and CD38$^+$ effector T cells after 72 h of culture. Results are pooled from 4 independent experiments. Data are expressed as means ± SEM. *p* values were calculated using an unpaired Student's *t* test. **c** Purified adult healthy control CD38^{neg} and CD38$^+$ effector T cells were stimulated for 72 h with anti-CD3/CD28 stimulation beads (0.5 bead per T cell). IFNγ and IL-17 production in supernatants was measured by ELISA. *p* values were calculated using a Student's *t* test. NS, not significant, $*p < 0.05$, $**p < 0.01$

CD38$^+$ effector T cells from peripheral blood of adult healthy individuals using flow cytometry. Frequencies of TIGIT$^+$, CTLA-4$^+$, and ICOS$^+$ cells were significantly increased in CD38$^+$ effector cells compared to CD38^{neg} effector T cells. The CD38$^+$ effector T-cell population contained 40% TIGIT$^+$ cells and 20% CTLA-4$^+$ cells versus only 20% and 10% respectively in the CD38^{neg} T-cell population (Fig. 3e). TIM-3 expression on CD4$^+$ T cells or CD38$^+$ effector T cells in peripheral blood was very low when analyzed by flow cytometry and therefore excluded from further analyses.

We next pursued to investigate whether this differential inhibitory profile was stable allowing it to be used for immune monitoring of intestinal tissue-localized responses. Thereto, we cultured purified CD38^{neg} and CD38$^+$ effector T cells from adult healthy individuals with allogeneic monocyte-derived DCs in an MLR and determined whether the differential profile was maintained during cellular activation. As has been reported previously, CTLA-4 was upregulated upon activation on both CD38^{neg} and CD38$^+$effector T cells causing a loss of differential expression between these two populations (Fig. 3f). In contrast, MLR culture did not enhance TIGIT expression in the CD38^{neg} population, while the TIGIT expression was steadily maintained on the CD38$^+$ effector cells resulting in a stable differential frequency of TIGIT expressing cells between CD38^{neg} and CD38$^+$ T-cell populations after activation (Fig. 3f). The same results were obtained when using anti-CD3/CD28 ligation as a mode of activation with no significant changes in frequencies of TIGIT on CD4$^+$ T cells after activation (Figure S6A). Virtually all TIGIT$^+$ cells in the CD38^{neg} and CD38$^+$ effector T-cell populations expressed CD45RO (Figure S6B). Thus, during mucosal homeostasis in adult healthy individuals circulating CD38$^+$ effector T cells contain a large population of cells stably expressing the inhibitory receptor TIGIT when compared to the CD38^{neg} effector T-cell population.

Inhibitory receptor TIGIT expression identifies circulating CD38$^+$ effector T cells with immunoregulatory properties

The stable TIGIT expression after in vitro activation suggested that it could be a putative marker for monitoring the IL-10-producing regulatory component of the CD38$^+$ effector T-cell population. We therefore further analyzed expression of inhibitory receptors and cytokine profiles by CD38$^+$TIGIT$^+$ effector T cells. In adult healthy individuals, TIGIT$^+$ cells within the CD38$^+$ effector T-cell population more often co-expressed a second inhibitory receptor such as programmed death 1 (PD-1) or CTLA-4 when compared to the CD38$^+$TIGIT^{neg} cells but did not preferentially co-express FoxP3 or CD25 (Fig. 4a). Cells co-expressing the costimulatory molecule ICOS were more frequent in CD38$^+$TIGIT$^+$ effector T cells when compared to CD38$^+$TIGIT^{neg} effector T cells (Fig. 4a).



In agreement with the inhibitory function of TIGIT, CD38$^+$TIGIT$^+$ cells contained higher frequencies of IL-10$^+$ cells after PMA-ionomycin stimulation compared to CD38$^+$TIGIT^{neg} cells (Fig. 4b). The association between TIGIT and IL-10 was specific for the CD38$^+$ effector T-cell population, as CD38^{neg}TIGIT$^+$ cells did not have higher frequencies of IL-10$^+$ cells compared to CD38^{neg}TIGIT^{neg} cells (Fig. 4c). CD38$^+$TIGIT$^+$ cells also contained lower frequencies of IFNγ secreting cells with a lower mean fluorescence intensity after PMA-ionomycin stimulation (Fig. 4b) and secreted lower amounts of IFNγ after anti-CD3/CD28 stimulation compared to CD38$^+$TIGIT^{neg} effector T cells (Fig. 4d). This agreed with high baseline levels of *IL10* mRNA expression and lower *IFNG*, *TNFA* and *IL17* mRNA expression in CD38$^+$TIGIT$^+$ cells (Fig. 4e). Importantly, the frequency of IL-10$^+$ cells in CD38$^+$TIGIT$^+$ cells was significantly higher than the frequency of IL-10$^+$ cells in CD38^{neg}TIGIT$^+$ cells ($p = 0.0027$, Student's t test). This demonstrates that TIGIT$^+$ cells in

Fig. 3 The inhibitory receptor TIGIT is strongly enriched on circulating healthy control CD38$^+$ effector T cells and its expression is maintained upon cellular activation. **a–d** RNA was isolated from adult healthy control CD38^{neg} and CD38$^+$ effector T cells (pre-gated on single CD3$^+$CD4$^+$CD62L^{neg} cells) purified by flow cytometric cell sorting populations from blood of three adult healthy individuals and differentially expressed genes were identified by RNA sequencing. **a** Number of differentially expressed genes. **b** Heatmap of all genes that were differentially expressed in CD38^{neg} and CD38$^+$ effector T cells (adjusted p value <0.05). **c** Log 2 fold change difference for gene subsets significantly upregulated in the CD38$^+$ effector T-cell population. **d** Validation of RNA-seq results with quantitative RT-PCR analysis. Log 2 fold change of known human regulatory genes in RNA-seq (white) and qRT-PCR (black). Data are represented as means + SEM. **e**, **f** Peripheral blood from adult healthy individuals ($n = 15$) was analyzed for expression of CD3, CD4, CD62L, CD38, TIGIT, CTLA-4, and ICOS by flow cytometry. **e** Frequencies of TIGIT$^+$, CTLA-4$^+$ and ICOS$^+$ cells in the total CD4$^+$ T-cell population, CD38^{neg} and CD38$^+$ T cells (pre-gated on CD4$^+$CD62L^{neg} cells). Data are expressed as median ± IQ range. p values calculated using a Mann–Whitney analysis. **f** Purified CD38^{neg} and CD38$^+$ T cells (pre-gated on CD4$^+$CD62L^{neg} cells) were cultured for 72 h with mature monocyte-derived DCs (ratio 10:1). Frequencies of CTLA-4$^+$ and TIGIT$^+$ cells at 72 h. Results are pooled from 4 independent experiments. Data are expressed as mean ± SEM. p values were calculated using a Student's t test. NS, not significant, ***$p < 0.001$

the CD38$^+$ effector T-cell population preferentially produce IL-10 when compared to TIGIT$^+$ cells in the CD38^{neg} effector T-cell population.

To test functional regulatory capacity of CD38$^+$TIGIT$^+$ effector T cells such as modulating DC activation, we cultured immature monocyte-derived DCs together with purified peripheral blood CD38$^+$TIGIT$^+$ or CD38$^+$TIGIT^{neg} effector T cells in the presence of anti-CD3 and lipopolysaccharide (LPS). Co-culture with CD38$^+$TIGIT$^+$ T cells yielded lower *IL12p35*, *IL23p19* and *IL6* mRNA expression in DCs when compared to co-cultures with CD38$^+$TIGIT^{neg} T cells (Fig. 4f). This effect was more pronounced for TIGIT$^+$ cells in the CD38$^+$ effector T-cell population compared to TIGIT$^+$ cells in the CD38^{neg} effector T-cell population (Fig. 4f). In contrast, TIGIT expression on CD38$^+$ effector T cells did not affect the maturation status of monocyte-derived DCs, as defined by surface expression of CD40, CD80, and CD86 (Figure S7). These data indicate that TIGIT expressing CD38$^+$ effector T cells from adult healthy individuals have the capacity to regulate inflammatory cytokine expression by monocyte-derived DCs. Overall, these demonstrate that TIGIT expression identifies a regulatory subpopulation of T cells within the CD38$^+$ effector T-cell pool.

Low frequencies of circulating CD38$^+$TIGIT$^+$ effector T cells identify pediatric IBD patients with reduced duration of clinical remission during follow up

We next assessed whether TIGIT expression on circulating CD38$^+$ effector T cells identified changes in regulatory responses in peripheral blood of pediatric IBD patients (Figure S8A). Strikingly, the frequency of TIGIT$^+$ cells in the CD38$^+$ effector T-cell population of patients with active IBD was drastically reduced in a subgroup of patients when compared to age-matched controls, with half of the patients having a TIGIT$^+$ cell frequency below 25% (Fig. 5a). Frequencies of TIGIT$^+$ cells in the total CD4$^+$ T-cell pool were not different compared to controls (Fig. 5a). Degree of TIGIT expression per cell (MFI) by CD38$^+$ effector T cells was not different between IBD patients and controls, nor between IBD patients with low versus high frequencies of TIGIT. Increased frequencies of activated CD45RA^{neg}CD25$^+$ cells inversely correlated with reduced frequencies of TIGIT$^+$ cells in the CD38$^+$ effector T-cell population (Fig. 5b), indicating that the regulatory component that is normally present in CD38$^+$ effector T cell population is decreased in a subgroup of IBD patients that has a high inflammatory component.

This raised the question whether TIGIT frequency classifies IBD heterogeneity prior to treatment. Crucially, a TIGIT percentage below 25% prior to treatment associated with a reduced duration of clinical remission (Fig. 5c and S8B). For comparison, attempting to achieve such classification on the basis of fecal calprotectin levels above or below 100, 250, or 1000 µg/g at time of diagnosis were not predictive of duration of remission (Fig. 5d, Figure S8C and S8D).

To investigate whether TIGIT frequency in the CD38$^+$ effector T-cell population is restored during therapy, we performed a follow up analysis of peripheral blood of pediatric IBD patients. Despite a wide range of immunosuppressive treatments, frequencies of TIGIT$^+$ cells significantly increased during therapy except in one patient, who did not achieve clinical remission at time of analysis (Fig. 5e). The on-treatment changes in TIGIT frequencies were exclusively confined to CD38$^+$ effector T cells, as such changes could not be observed in total CD4$^+$ T cells or CD38^{neg} effector T cells (Fig. 5e).

Taken together, TIGIT frequencies in CD38$^+$ effector T cells are reduced in a subgroup of pediatric IBD patients that are at risk for early disease relapse.

DISCUSSION

We demonstrate that analysis of circulating CD38$^+$ effector T cells, a population that is enriched for cells with mucosal antigen specificity, instead of total CD4$^+$ T cells, lowers the threshold for detection of changes in regulatory versus inflammatory T-cell responses. We observed that circulating CD38$^+$ effector T cells of healthy individuals have a predominant regulatory TIGIT$^+$CD38$^+$ effector T-cell subpopulation strongly enriched in expression of inhibitory molecules and IL-10. In peripheral blood of pediatric IBD patients with active disease, the phenotype of circulating CD38$^+$ effector T cells, but not total CD4$^+$ T cells, was altered, with increased frequencies of activated inflammatory CD25$^+$CD45RA^{neg} and decreased frequencies of regulatory TIGIT$^+$ cells. Low frequencies of TIGIT$^+$ cells (<25%) in CD38$^+$ effector T cells identified a subgroup of IBD patients at diagnosis that had reduced duration of clinical remission during follow up. In patients responding to immunosuppressive treatment, frequencies of TIGIT and CD25 in CD38$^+$ effector T cells of IBD patients normalized to frequencies similar to that seen in CD38$^+$ effector T cells of age-matched healthy individuals.

IBD is a complex disease with a high degree of clinical heterogeneity and as a result an unpredictable response to therapy. Therefore, it is crucial to develop biological parameters to predict disease activity, disease course, and response to treatment, enabling the design of tailored treatment strategies. As inflammatory CD4$^+$ T cells drive IBD pathology, monitoring the inflammatory status of T cells in peripheral blood is highly desired to differentiate patients with a severe versus a mild disease course. It has, however, not been possible to date to monitor tissue-localized T-cell responses in peripheral blood of IBD patients. We are the first to show that phenotypic changes in peripheral blood CD38$^+$ effector T cells instead of total CD4$^+$ T cells reflect tissue-localized disease in IBD patients. In particular, IBD patients with active disease have increased frequencies of activated CD25$^+$CD45RA^{neg} cells and decreased frequencies of TIGIT$^+$ but not FoxP3$^+$ cells compared to age-matched healthy controls. This increased frequency of activated CD38$^+$ effector T cells was observed in both CD and UC patients (Figure S2). Crucially, heterogeneity in the percentage of TIGIT$^+$ cells in CD38$^+$ effector T-cell population prior to treatment differentiated patients with

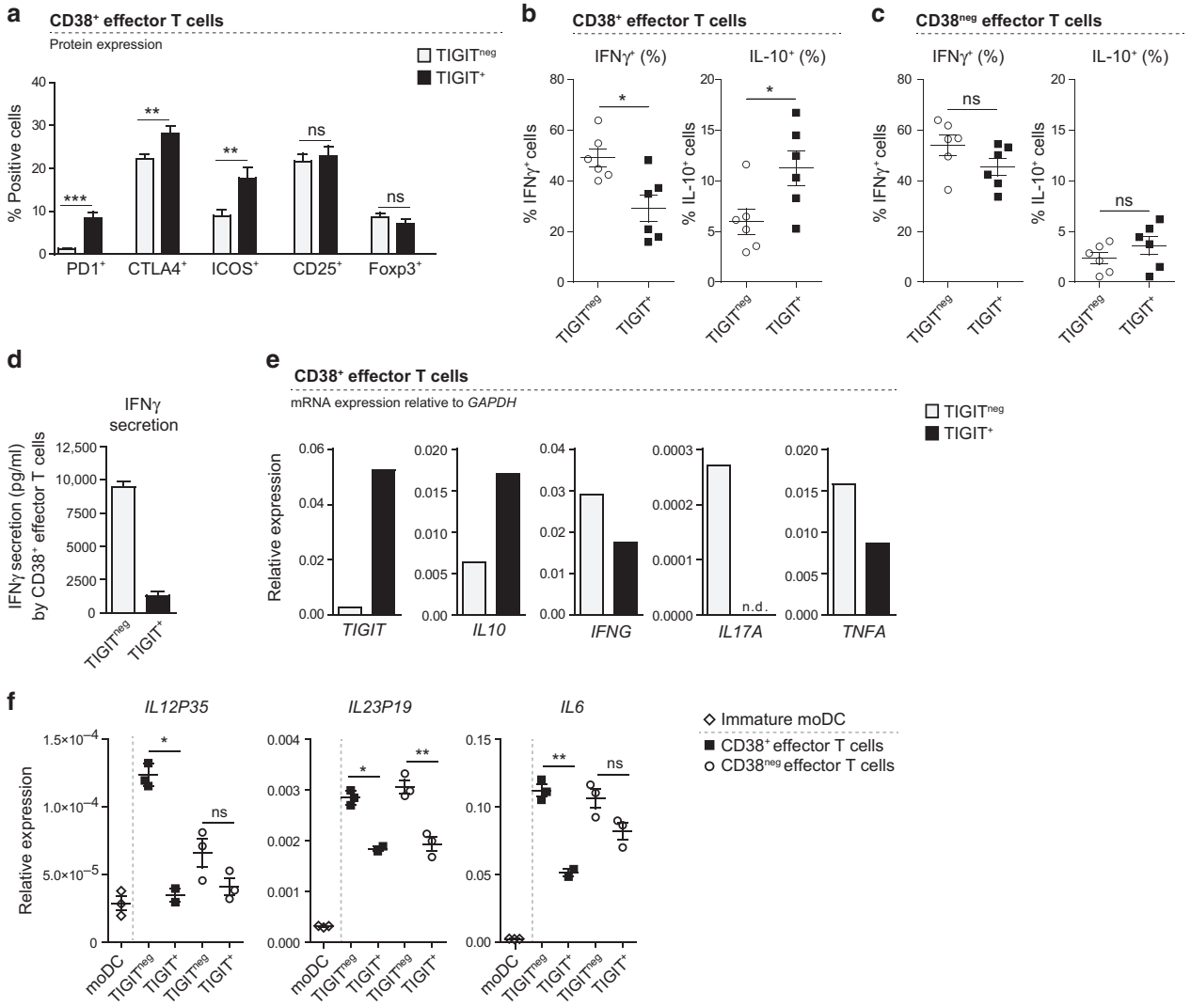


Fig. 4 Inhibitory receptor TIGIT expression identifies circulating CD38$^+$ effector T cells with immunoregulatory properties. **a** Flow cytometric analysis of TIGIT^{neg} and TIGIT$^+$ in CD38$^+$ effector T-cell population (pre-gated on CD4$^+$CD62L^{neg} cells) of adult healthy individuals ($n = 15$). Frequencies of cells positive for PD-1, CTLA-4, ICOS, Ki67, CD25 and FoxP3 are shown. **b–c** CD4$^+$CD62L^{neg} T cells were obtained from the blood of adult healthy individuals ($n = 6$) and stimulated for 4.5 h with PMA plus ionomycin in the presence of Brefeldin A. IFNγ$^+$ cells and IL-10$^+$ cells were determined in TIGIT^{neg} and TIGIT$^+$ cells within CD38$^+$TIGIT^{neg}, CD38$^+$TIGIT$^+$, CD38^{neg}TIGIT^{neg}, and CD38^{neg}TIGIT$^+$ T-cell populations. **d** TIGIT^{neg} and TIGIT$^+$ cells were purified from the CD38$^+$ effector T-cell population (pre-gated on CD4$^+$CD62L^{neg} cells) from the peripheral blood of adult healthy individuals and stimulated for 14 h with anti-CD3/CD28 stimulation beads (0.5 bead per T cell). IFNγ release in supernatants was measured by ELISA. Representative of two independent experiments. **e** Purified adult healthy control TIGIT^{neg}CD38$^+$ and TIGIT$^+$CD38$^+$ T cells analyzed for *TIGIT*, *IL10*, *IFNG*, *IL17*, and *TNFA* mRNA expression relative to *GAPDH* by quantitative PCR. Representative of three independent experiments. **f** Autologous immature monocyte-derived DCs were cultured together with purified CD38$^+$TIGIT^{neg}, CD38$^+$TIGIT$^+$, CD38^{neg}TIGIT^{neg} or CD38^{neg}TIGIT$^+$ effector T cells for 24 h in the presence of anti-CD3 (1 μg/ml) and LPS (10 ng/mL) in a ratio 2:1 (T cell: monocyte-derived DC). Analysis of inflammatory cytokines *IL12p35, IL23p19,* and *IL6* mRNA expression relative to *GAPDH* by quantitative PCR. One representative experiment shown out of two independent experiments. Data are represented as means ± SEM. *p* values were calculated using a Kruskal–Wallis H analysis. NS, not significant, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

reduced duration of clinical remission during follow up. Such differentiation was purely based on compositional changes of the CD38$^+$ effector population as the total number of CD38$^+$ effector T cells, the white blood cell count as well as the frequencies of CCR9$^+$ and α4β7$^+$ expressing cells within the CD38$^+$ population, did not differ between IBD patients and controls (Figure S1B and S3).[8] The importance of analysis of the CD38$^+$ effector T-cell population instead of total CD4$^+$ T cells is further emphasized by a previous study that could not detect alterations in circulating TIGIT$^+$ cells when monitoring total CD4$^+$ T cells in IBD patients.[8,13]

TIGIT has not been previously implicated in intestinal adaptive immune homeostasis. In healthy individuals 40% of peripheral blood CD38$^+$ effector T cells expressed TIGIT and were enriched in inhibitory receptors and IL-10 expression, reflecting a preferential regulatory phenotype of these mucosally-imprinted T cells. TIGIT expression was stable during in vitro activation and proliferation of TIGIT$^+$ cells (Figure S9). Although previous findings established a role for TIGIT in Treg-mediated immune suppression[14], we found the majority of CD38$^+$ effector T cells to express TIGIT but not FoxP3. Our findings are in line with data showing TIGIT, but not FoxP3, is positively correlated with IL-10 expression by CD4$^+$ T cells in models investigating tolerance induction in auto-immunity.[15] Likewise, we report changes in TIGIT but not FoxP3 in CD38$^+$ effector T cells in IBD patients. In line with previous data showing that TIGIT-Fc modifies DC cytokine production[16], we show that TIGIT

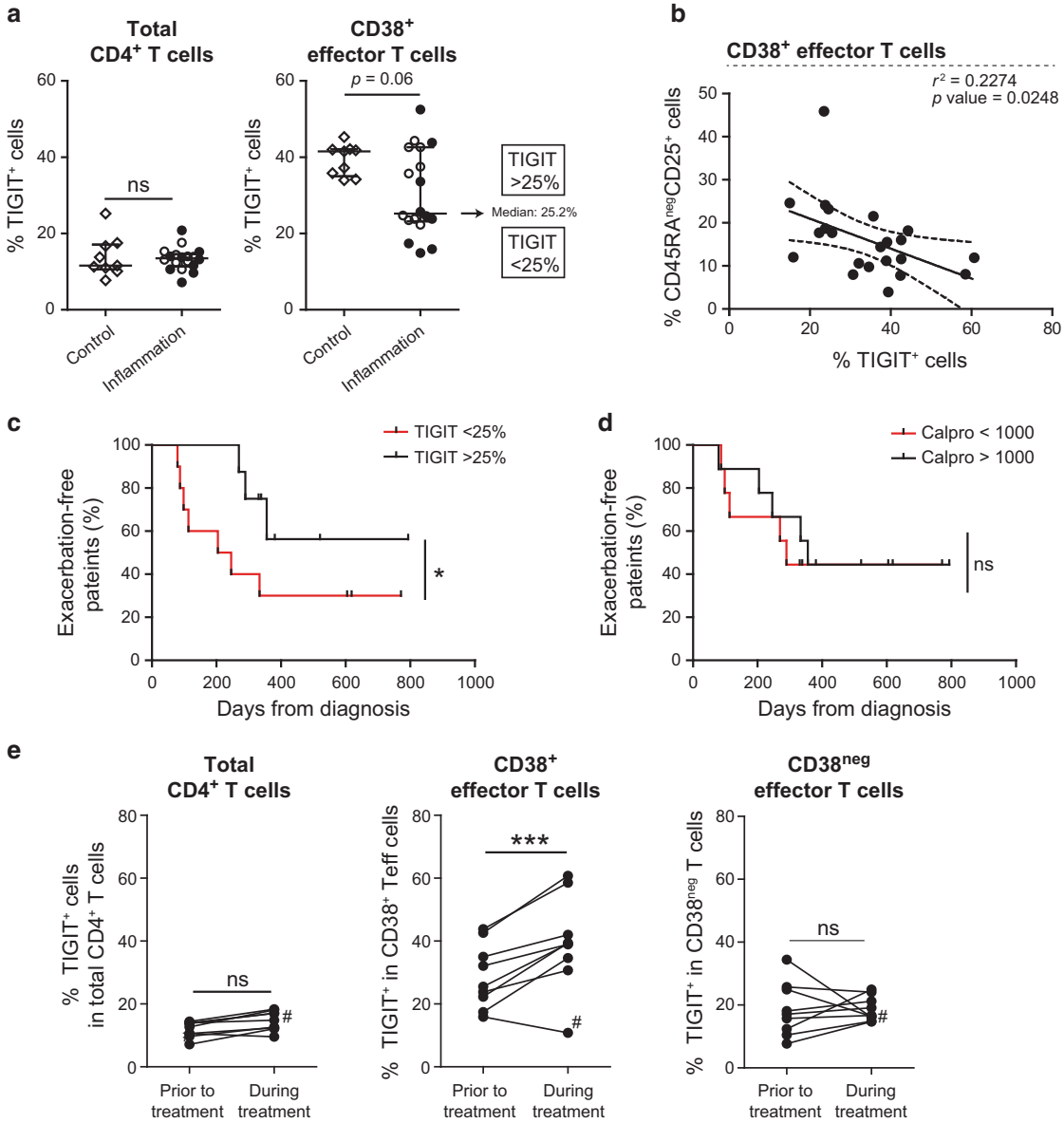


Fig. 5 Low frequencies of circulating CD38$^+$TIGIT$^+$ effector T cells identify pediatric IBD patients with reduced duration of clinical remission during follow up. Peripheral blood from pediatric IBD patients with active disease ($n = 18$, cohort II) and age-matched healthy controls ($n = 9$) was analyzed by flow cytometry. Open and closed symbols depict UC and CD patients respectively. **a** Frequency of TIGIT$^+$ cells in the total CD4$^+$ T-cell pool and the CD38$^+$ effector T-cell population. Data are expressed as median ± IQ range. *p* values were calculated using a Mann–Whitney analysis. **b** Correlation of the frequency of activated CD45RA^{neg}CD25$^+$ cells and TIGIT$^+$ cells in the CD38$^+$ effector T-cell population of pediatric IBD patients. **c** Kaplan–Meier overall exacerbation-free disease course for low versus high TIGIT frequencies on CD38$^+$ effector T cells. **d** Kaplan–Meier overall exacerbation-free disease course for fecal calprotectin levels (TIGIT < 25%, $n = 10$, median follow up of 618 days; TIGIT > 25%, $n = 8$, median follow up of 509 days; NS). **e** Frequency of TIGIT$^+$ cells in the total CD4$^+$ T-cell pool, CD38$^+$ effector T-cell population and CD38^{neg} effector T-cell population in peripheral blood of pediatric IBD patients at disease diagnosis (prior to treatment) and during immunosuppressive treatment (median treatment duration 223 days; IQ range 169–309). #indicates a patient not in remission during the follow up analysis and who was subsequently started on a 4 week steroid regimen. *p* values were calculated using a Wilcoxon signed rank test. NS, not significant, $^*p < 0.05$, $^{***}p < 0.001$

expressing CD38$^+$ effector T cells have the capacity to modulate inflammatory cytokine expression by monocyte-derived DCs, suggesting that TIGIT expressing cells may have a local immunomodulatory effect on surrounding cells when reaching the intestinal tissue.

It is striking that in healthy individuals, with a balanced mucosal tolerance to microbiota, CD38$^+$ effector T cells are enriched in other immune inhibitory receptors such as CTLA-4 and TIM-3 when compared to CD38^{neg} effector T cells. We chose to further analyze TIGIT because of the high percentage of CD38$^+$ effector T cells that expressed TIGIT whereas we were unable to identify circulating CD38$^+$ effector T cells that expressed surface TIM-3 when analyzed ex vivo, which is consistent with previous observations by others.[17] Moreover, in contrast to CTLA-4, TIGIT expression was stable upon differentiation and did not show transient upregulation in antigen experienced cells stimulated with anti-CD3. However, we do not exclude that analysis of multiple additional activational and regulatory markers on CD38$^+$ effector T cells may further enable IBD patient stratification prior to treatment.

To conclude, we provide the first evidence that tracing the composition of the circulating CD38$^+$ effector T-cell population of IBD patients allows to detect changes in the CD4$^+$ T-cell response that correlate with disease course and therapy responsiveness. The identification of CD38$^+$ effector T cells as targets for tracing chronic inflammation has immediate relevance for clinical trials in IBD but may be further exploited in other diseases such as graft versus host disease and checkpoint inhibitor-induced inflammation in the setting of cancer.

MATERIAL AND METHODS

Patients

Two cohorts of pediatric IBD patients were investigated. Cohort I consisted of pediatric IBD patients in clinical remission who visited the outpatient clinic ($n = 26$) and pediatric patients who underwent a colonoscopy with suspicion of IBD. After diagnosis, those with biopsy-proven active IBD were included in the analyses and termed "inflammation" ($n = 22$). In cohort I, remission was defined by physician global assessment (PGA). Cohort II consisted of treatment-naïve patients with biopsy-proven active IBD termed "inflammation" ($n = 18$). Of this group, peripheral blood of 9 patients was also analyzed during follow up after initiation of treatment. In cohort II, clinical remission and exacerbation were defined using PGA and clinical disease scores. Age-matched controls without any inflammatory or intestinal disease who underwent orthopedic surgery at the time of blood withdrawal ($n = 22$) were included in the control group. Peripheral blood was also obtained from adult healthy controls ($n = 25$). The Medical Ethical Committee of the Erasmus University Medical Centre-Sophia Children's Hospital Rotterdam approved this study (METC 2007-335). Written informed consent was obtained from every patient and parents before study inclusion. Additional patient characteristics are shown in Figure S1 and Figure S6.

T-cell isolation and T-cell/DC co-cultures

Venous blood was collected in EDTA tubes and peripheral blood mononuclear cells (PBMCs) were isolated using a Ficoll-Hypaque gradient according to standard protocol (Axis-Shield). PBMCs were stained for flow cytometry and CD3$^+$CD4$^+$CD62L^{neg} T cells were sorted into CD38^{neg} and CD38$^+$ effector T-cell populations by flow cytometric cell sorting on a BD FACS Aria (BD Biosciences). Purified T-cell populations were stimulated with anti-CD3/CD28 stimulator beads or co-cultured with allogeneic LPS-stimulated monocyte-derived dendritic cells (DCs) for 72 h.

CD14$^+$ monocytes were isolated using CD14 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany) with a positive selection method according to the manufacturer's instructions. Purified monocytes (>90%) were differentiated into monocyte-derived DCs during a 6-day culture with 800 U/ml GM-CSF (Novartis, Basel, Switzerland) and 400 U/ml IL-4 (R&D). After harvesting on day 6, 1×10^4 monocyte-derived DCs were matured with LPS (100 ng/ml) for 24 h. On day 7, supernatants were removed and the matured monocyte-derived DCs were co-cultured with 1×10^5 purified T cells. Cells were cultured in Iscove's modified Dulbecco's medium (Lifetechnologies, Grand Island, NY, USA) supplemented with heat inactivated fetal calf serum, Glutamax (Lifetechnologies), 2-mercaptoethanol, penicillin and streptomycin.

In some experiments, CD4$^+$ T cells were isolated from PBMCs by negative selection using the Dynabeads Untouched Human CD4$^+$ T cell kit (11346D, Thermo Fisher). The unlabeled CD4$^+$ T cells were separated into CD62L^{neg} and a CD62L$^+$ cell fractions by using CD62L MicroBeads (Miltenyi Biotec). 2×10^5 CD62L^{neg} T cells were stimulated for 4.5 h with phorbol 12-myristate 13-acetate (PMA, 0.02 µg/ml, Sigma-Aldrich) and ionomycin (0.5 µg/ml, Sigma-Aldrich) in the presence of Brefeldin A (3 µg/ml eBiosciences) for the last 3.5 h and subsequently analyzed for intracellular cytokine expression.

Flow cytometry

After erythrocyte lysis, whole blood samples were stained for flow cytometry using monoclonal antibodies against CD3 (UCHT1, BD, or HIT3a, Biolegend), CD4 (SK3, BD), CD38 (HIT2, BD), CD62L (DREG-56, Biolegend), CD25 (2A3, BD), CD45RA (HI100, BD), CD45RO/RPE (UCHL1, Dako), TIGIT (MBSA43, eBiosciences), ICOS (C398.4 A, Biolegend), CD127 (HIL-7R-M21, BD), PD1 (MIH4, eBiosciences) and HLA-DR (L243, BD). Intracellular staining was performed with the FoxP3 fixation and permeabilization staining buffer kit, according to manufacturer's protocol (eBiosciences), followed by staining with anti-FoxP3 (236 A/E7 or PCH101, eBiosciences), anti-Helios (22F6, Biolegend), anti-Ki67 (20Raj1, eBiosciences) or anti-CTLA-4 (BNI3, BD) and the appropriate isotype controls.

For intracellular cytokine staining after culture, cells were stimulated for 4.5 h with PMA and ionomycin in the presence of Brefeldin A as described above. After incubation with antibodies directed to surface proteins, cells were fixed in 2% formaldehyde and permeabilized with saponin (Sigma-Aldrich), and labeled with antibodies to IL-10 (JES3-19F1, BD), IL-17A (eBio64DEC17, eBiosciences), IFNγ (4 S.B3, BD), or appropriate isotype controls (eBiosciences). Flow cytometric analysis was performed on a FACSCanto^{TM}II (BD Biosciences).

Cytokine analysis

Cytokine concentrations in cell supernatants were analyzed using an enzyme-linked immunosorbent assay set for IFNγ (eBiosciences), IL-10 (eBiosciences), and IL-17 (R&D Systems) according to the manufacturer's instructions.

RNA extraction and Illumina library preparation

Total cellular RNA from purified T cells was extracted using the NucleoSpin® RNA-XS extraction kit for the isolation of RNA (Macherey-Nagel) according to manufacturer instructions. RNA levels, quality and purity were assessed with the RNA 6000 Nano assay on the Agilent 2100 Bioanalyzer.

cDNA was synthesized using a mix of random hexamers (2.5 µM), oligo(dT) primers (20 nM), dNTP (0.2 mM), M-MLV (200 units, Promega) and RNAsin (25 units, Promega). The samples used for transcriptome analysis were amplified using SMARTer Ultra Low RNA kit (Clontech Laboratories) following the manufacturer's protocol. Amplified cDNA was further processed according to TruSeq Sample Preparation v2 Guide (Illumina) and paired end sequenced on the HiSeq 2500 (Illumina, San Diego, California; 76 cycles, paired end sequencing, rapid run, 3 samples per lane). Human transcripts were aligned to the RefSeq transcriptome and analyzed with DESeq2. Fragments per kilobase of transcript per million mapped reads (FPKM) values were calculated using Cufflinks.

Quantitative PCR

A maximum of 1000 ng mRNA was used for the synthesis of cDNA. Real-time quantitative PCR was performed using SYBR Green on an AbiPrismR 7900 Sequence Detection system (PE Applied Biosystems, Foster City, CA). The relative expression to *GAPDH* for each gene was measured as $2^{(-\Delta Ct)}$. Primer sets used were: *GAPDH, Fw 5′-GTCGGAGTCAACGGATT-3′ Rv 5′-AAGCTTCCCGTTCTCAG-3′; TIGIT, Fw 5′-TTGGGGTGGCACATCT-3′ Rv 5′- CGACCACCACGATGACT-3′; IFNG, Fw 5′-CCAGGACCCATATGTAAAAG-3′ Rv 5-TGGCTCTGCATTATTTTTC-3′; IL10, Fw 5′-CCCCAAGCTGAGAACC-3′ Rv 5′-ACGGCCTTGCTCTTGT-3′; CTLA4, Fw 5′-GCTTGCCTTGGATTTCA-3′ Rv 5′-GCCGCACAGACTTCAGT-3′; ICOS, Fw 5′-CTGGCAAACATGAAGTCAG-3′ Rv 5′-CACCTCCGTTGTGAAATATAA-3′; CD38, Fw 5′-GGCCCATCAGTTCACAC-3′ Rv 5′-GAAACCGTTTTCCAGAATACT-3′; IL6, Fw 5′-CCCCCAGGAGAAGATTC-3′, Rv 5′-GCTGCTTTCACACATGTTACT-3′; IL17A, Fw 5′-GAAGGCAGGAATCACAATC-3′ Rv 5′-GCCTCCCAGATCACAGA-3′; TNFA, Fw 5′-CGCTCCCCAAGAAGAC-3′ Rv 5′-GGTTCGAGAAGATGATCTGA-3′; IL12P35, Fw 5′-CTGGCCTCCAGAAAGAC-3′ Rv 5′-GTGGCACAGTCTCACTGTT-3′; IL23P19, Fw 5′-CAGGGACAACAGTCA*

GTTCT-3′ Rv 5′-CTGCGAAGGATTTTGAAG-3′; CD80, Fw 5′-TGGGCCATTA CCTTAATCT-3′ Rv 5′-TCTGCGGACACTGTTATACA-3′; CD86, Fw 5′-TG GGGTCATTTCCAGATA-3′ Rv 5′-GTGCGGCCCATATACTT-3′; CD40 Fw 5′-T TGGGGTCAAGCAGATT-3′ Rv 5′-CCTGGGGACCACAGAC-3′.

Statistical analysis

Baseline demographic and disease characteristics were evaluated for the entire cohort using descriptive statistics, including means and standard deviations (SD) or median and interquartile ranges (IQ range) for continuous variables, and frequencies and percentages for categorical outcomes. Significance between two groups was determined using Student's *t*-test or Mann–Whitney *U*-test and differences between multiple groups by using the Kruskal–Wallis H test. The Wilcoxon signed-rank test was performed when samples were paired. p Values of <0.05 were considered statistically significant. Prism software (GraphPad Software, Version 5.0, La Jolla, CA) was used for all statistical analysis.

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AUTHOR CONTRIBUTIONS

M.J. and J.S. contributed to study concept and design, interpretation of data, and drafting of the manuscript. M.J. designed, conducted, and analyzed experiments. R.H., T.C., and S.V. contributed to sequencing data analysis and interpretation. C.M., L.F.dR., H.R., L.vB., Y.S., and F.M. performed experiments and data analysis. R.H., T.C., and S.V. provided important intellectual content. L.dR. and J.C.E. contributed important intellectual content and contributed the patient-sample collection. J.S. conceived the project, obtained funding and had full responsibility for the study.

ADDITIONAL INFORMATION

The online version of this article (https://doi.org/10.1038/s41385-018-0078-4) contains supplementary material, which is available to authorized users.

Competing interests: The authors declare no competing interests.

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