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Th17/Treg ratio in human graft-versus-host disease

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Abstract

Th17 cells have never been explored in human GvHD. We studied the correlation between the presence of Th17 cells in the gastro-intestinal (GI) tract and in the skin with histological and clinical parameters. We first analyzed a cohort of 40 patients with suspected GvHD of the GI tract. TNF, TNF receptors, and Fas expression, and apoptotic cell, $CD4^+$ $IL\text{-}17^+$ cells (Th17) and $CD4^+$ $Foxp3^+$ cells (T_{reg}) were quantified. A Th17/T_{reg} ratio<1 correlated both with the clinical diagnosis (p<0.001), and ≥ 2 pathologic grade (p<0.001). A Th17/T_{reg} ratio<1 also correlated with the intensity of apoptosis of epithelial cells (p=0.03), Fas expression in the cellular infiltrate (p=0.003), TNF and TNF receptors expression (p<0.001). We then assessed Th17/T_{reg} ratio in two other independent cohorts; a second cohort of 30 patients and confirmed that Th17/T_{reg} ratio<1 correlated with the pathological grade of GI GvHD. Finally 15 patients with skin GvHD and 11 patients with skin rash but without pathological GvHD were studied. Results in this third cohort of patients with skin disease confirmed those found in patients with GI GvHD. These analyses in 96 patients suggest that Th17/T_{reg} ratio could be a sensitive and specific pathological in situ biomarker of GvHD.

MESH Keywords Adult ; Cohort Studies ; Female ; Graft vs Host Disease ; immunology ; metabolism ; Hematologic Neoplasms ; immunology ; metabolism ; therapy ; Humans ; Interleukin-17 ; immunology ; metabolism ; Male ; Middle Aged ; Prognosis ; Skin Diseases ; immunology ; metabolism ; therapy ; T-Lymphocytes, Regulatory ; immunology ; metabolism ; Tumor Necrosis Factor-alpha ; metabolism

Introduction

Graft versus host disease (GvHD) involves dysregulation of inflammatory cytokine cascades and distorted donor cellular response against host alloantigens (reviewed in 1). The characterization of $CD4^+$ /interleukin 17 (IL-17)-secreting subset (Th17) and of the regulatory $CD4^+$ /$Foxp3^+$ cell (T_{reg}) has had a major impact on our understanding of immune processes 2 –6 . Th17 and T_{reg} might contribute to human autoimmune diseases, including inflammatory bowel disease (IBD) 2 –5 . Experimental data suggest a reciprocal relationship between Th17-induced pathology, and T_{reg} regulatory role. In murine models, Th17 cells induce autoimmunity through tissue inflammation promotion, and innate immune system mobilization 2 –5 . However, in the gut Th17 cells might also have modulatory and protective roles. Murine models provide recent controversial results on the role of Th17 in GvHD 4 , 7 –11 . We are not aware of any published data on Th17 cells in human GvHD. To investigate their potential implication in GvHD-induced inflammation, we studied Th17 and T_{reg} in 3 independent cohorts of patients (n=96) who had gut or skin biopsies and their relationship with histological and clinical parameters.

Patients and methods

Patients

Our primary aim was to study Th17 and Treg populations in the setting of gastro-intestinal (GI) GvHD. Biopsies were performed as diagnostic procedures for digestive symptoms. Clinical grading used the 1994 consensus conference criteria 12 and pathological were

performed as previously described 13 . Hospital Saint Louis ethical review board approved the design of this study. Patients with digestive symptoms underwent endoscopy and biopsies before any treatment with steroids. We first took advantage of a first study 13 in which patients biopsies have been extensively studied for Fas, TNF and apoptosis pathways to search for Treg and Th17 infiltration and association with Fas and TNF pathways (if any). Then, we aimed to confirm results of Treg and Th17 localization in an independent cohort of patients with GI GvHD and search for a role (if any) of the intensity of the conditioning regimen. Finally we studied these cell subsets in patient with skin GvHD. Thus, 3 different cohorts of patients (total n=96) were sequentially studied:

- 40 out of 78 patients from the previously published cohort had available duodenal biopsies. Median age was 34 years. Conditioning regimens (all myeloablative) included total body irradiation (TBI) and cyclophosphamide (n=13), or associated busulfan (Bu) and cyclophosphamide (n=15). Acute lymphoblastic leukemia patients received TBI or Bu with melphalan and cytarabine (n=9). Aplastic anemia patients received cyclophosphamide and anti-thymocyte globulins. Cyclosporine and methotrexate were used for most patients (n= 28). Patients underwent duodenal biopsies for unexplained nausea or vomiting (n= 9) or stage 1–2 diarrhea (n=31).

- A second cohort of 30 patients with GI biopsy was studied to confirm in an independent series biological results in GI-GvHD. It included:

- 11 patients (4 males, 7 females; median age 30 years) with pathological GI GvHD who underwent an allogeneic SCT after myeloablative conditioning. Ten patients were grafted for hematological malignancies. All had stage 2 clinical lower GI GvHD at the time of biopsy.

- 10 patients (6males, 4 females; median age 48 years) who underwent SCT after a reduced intensity conditioning that included fludarabine and 2Gy TBI in 6 patients (according to the Seattle's regimen) and low dose IV busulfan fludarabine and ATG in 4 patients (according to the MD Anderson's regimen). All 10 patients with hematological malignancies had also biopsy-proven GI GvHD (8 patients had stage 1 and 2 patients had stage 2 lower GI GvHD at the time of biopsy).

- Finally, 9 patients who had no evidence of pathological GvHD at the time of upper GI biopsy were used as controls in this second cohort. These patients (6 males, 3 females; median age 30 years) underwent SCT for hematological malignancies either after a myeloablative (n=5), or a Fludarabine + 2Gy TBI reduced intensity conditioning (n=4) (4 patients had stage 1 lower GI GvHD at the time of biopsy and 5 patients had unexplained nausea/vomiting).

- Finally, a 3rd cohort of 26 patients with skin rash was studied. This series included 10 patients with biopsy-proven acute GVHD, 5 patients with evidence of pathological chronic GvHD and 11 patients without evidence of GvHD after pathological examination of skin biopsies. The 10 patients (7 males, 3 females; median age 34 years) with acute GvHD all underwent SCT for hematological malignancies after a myeloablative conditioning regimen. Four out of the 5 patients with chronic GvHD (3 males, 2 females, median age 43 years) also received a myeloablative conditioning, and all 5 had acute leukemia. Finally among the 11 patients (7 males, 4 females; median age 36 years) without pathological skin GvHD, 9 underwent SCT for malignant diseases and 2 for Fanconi anemia.

Methods

Duodenal biopsies during fiber-optic examination and skin biopsies were performed, as previously described 13 –14 . All biopsies were performed before any steroid treatment, and for GI biopsies two of them used for systematic viral or fungal infection detection. Histological digestive and skin GVHD grading was done as previously described 13 .

TUNEL assay and immunohistochemistry were performed on frozen sections using CD45RA (Dako, Denmark), CD68 (Dako, Denmark), CD95 (Pharmingen, Germany), TNF (Genzyme, United Kingdom), and TNF receptors, TNFr55 and TNFr75 (Ig-G monoclonal antibodies kind gift from MBrockaus, Switzerland) as primary antibodies. Double immunostainings were performed with Ventana Discovery reagents (Ventana, Arizona). For Foxp3/CD4 or Foxp3/CD8 incubation with monoclonal mouse antibody against human Foxp3 (clone22510, Abcam; dilution1:50), detected by UltraMap detection kit, was followed by incubation with mouse monoclonal antibody against human CD4 (clone4B12, Ménarini; dilution1:20) or with monoclonal antibody against human CD8 (cloneCD8/144B, Abcam; dilution1:100) detected by FastRed detection kit. For IL-17/CD4 double immunostaining, incubation with polyclonal rabbit antibody against IL-17 (H-132sc-7927, Santa-Cruz; dilution1:100), detected by UltraMap detection kit, was followed by an incubation with a mouse monoclonal antibody directed against human CD4 (clone 4B12, Ménarini; dilution 1:20), detected by FastRed detection kit. Endogenous peroxidase inhibition and nonspecific binding sites blocking were systematically performed. Controls with irrelevant isotypic antibodies, and absence of primary antibody were systematically performed. Double immunofluorescent staining was performed on frozen sections for Foxp3/CD4, IL-17/CD4 and Foxp3/IL-17. Primary antibodies were covalently linked to Alexafluor 488 or Alexafluor 594 using APEX Antibody Labeling Kits (Invitrogen). Sections were incubated in PBS pH 7.4 containing 5% bovine serum albumin for 30 min at room temperature (RT). Monoclonal mouse antibody to CD4 (clone 4B12, Ménarini; dilution 1:20), monoclonal mouse antibody to Foxp3

(clone 22510, Abcam; dilution 1:50), or polyclonal rabbit antibody to IL-17 (H-132 sc-7927, Santa-Cruz; dilution 1:100) were applied to sections for 1 hour at RT. Sections were finally mounted in Vectashield medium containing DAPI. CD4 Th17/CD4 T_{reg} ratio was expressed as median and inter-quartile range (IQR) of the number of cells per field at 400× magnification.

Examiners were aware of clinical signs and treatment (if any) of the patients at the time of biopsies and for routine GvHD pathological evaluation. However, for Th17/Treg stainings patients were identified by anonymous 7 digits code corresponding to laboratory identification. It is thus unlikely that one examiner could associate patient identification with biopsy code. More importantly, biopsies were independently evaluated by 2 examiners (PR and AJ). In all cases of disagreement between examiners, a common reading was organized to achieve a consensus on count.

Flow cytometry and intracellular cytokine staining

Presence in the peripheral blood mononuclear cells (PBMCs) of $CD3^+$ $CD4^+$ IL-17^+ T cells (Th17) and $CD3^+$ $CD4^+$ $Foxp3^+$ $CD25^{high}$ (Treg) was evaluated by flow cytometry. PBMCs were separated by density gradient centrifugation with lymphocytes separation medium (Organon, Durham, NC). After cell surface staining, cells were washed and resuspended in fixation/permeabilization solution (BD, Cytofix/Cytoperm kit; BD pharmingen) and intracellular staining was performed, following the manufacturer's instructions. For the detection of IL-17, PBMCs were incubated for 4 hours with 50 ng/mL phorbol myristate acetate and 750ng/mL ionomycin in the presence of monensin (eBioscience) in tissue culture incubator at 37°C.

Conjugated monoclonal antibodies for human CD3 (clone SK7) and CD4 (clone RPA-T4), were purchased from BD Biosciences. Conjugated monoclonal antibodies for human CD25 (clone BC96), Foxp3 (clone PCH101), and IL-17 (clone eBio64DEC17), were purchased from eBioscience. Monoclonal antibodies were conjugated to either fluorescein isothyocyanate (FITC), phycoerythrin (PE), PE-Cyanin 5 (PE-Cy5), allophycocyanin (APC), allophycocyanin-7 (APC-Cy7), Pacific Blue and Amcyan. Stained cells were all analyzed by a BD LSR II flow cytometer and data were analyzed by FlowJo software (Tree Star).

Statistical analyses

Reproducibility of Th17 and T_{reg} counts was assessed through examination of discrepancy levels between the 2 examiners and intraclass correlation estimate with 95% confidence interval 15 . Th17 or T_{reg} characteristics (cell numbers or their ratio) were summarized through median level and range. Their ability to predict the existence of a clinical or pathological grade ≥2 GvHD was assessed through: Sensitivity , i.e. proportion of biopsies with a characteristic lower than a given limit in patients with acute clinical or pathological grade ≥2 GvHD. Specificity , i.e. proportion of biopsies with a characteristic equal to or higher than the same limit in patients without acute clinical or pathological grade ≥2 GvHD. The ability of a characteristic to predict clinical or pathological grade ≥ 2 GvHD was tested through 2-sided Fisher exact test, as well as association between Th17/T_{reg} ratio with other GvHD pathological markers. For the comparison on proportion of $CD4^+$ $CD25^{high}$ $Foxp3^+$ cells, of Th17 cells and Th17/T_{reg} ratio between the control group and the group with No GvHD on one hand, between the group with No GvHD and the group with acute GvHD on the other hand, Mann-Whitney non parametric test was used. The same test was used to compare median levels of $CD4^+$ cell counts, proportion of $CD4^+$ cells expressing Foxp3 or IL-17 as a function of pathological GvHD grade (0–1 versus 2 or more).

Results

Among 100 biopsies evaluated by the 2 examiners (PR, AJ), 96 counts showed a difference of 1 (n=21) or no difference at all (n=75) for Th17 identified by CD4/IL-17 double staining (Figure 1A,B). For T_{reg} identified by CD4/Foxp3 double staining (Figure 1C,D), the corresponding figures were 89, 20 and 69, respectively. Differences were never greater than 2 for Th17 and 3 for T_{reg} (3 biopsies). It should be noted that for the 11 discrepancies in Treg evaluation, 10 were for counts above 5. To summarize the high level of reproducibility between examiners, intraclass correlation was estimated to be 0.950 (95% confidence interval 0.927–0.966) for Th17 and 0.937 (95% confidence interval 0.907–0.957) for Treg.

Forty patients with suspected digestive GvHD (nausea/vomiting or diarrhea) had biopsies before any steroid treatment (1^{st} cohort); 18 had severe pathologically proven GvHD (pathological stage ≥ 2) (Table 1A). Whatever the severity of acute GvHD, the number of $CD4^+$ cells did not varied (p=0.63, Figure 2A). As identified by CD4/Foxp3 staining, an increased proportion of $CD4^+$ cells expressing Foxp3 in duodenal biopsies of patients with grade ≥ 2 compared with patients with grade <2 was observed (p<0.0001, Figure 2B). Conversely, a decreased proportion of $CD4^+$ cells expressing IL-17 identified by and CD4/IL-17 staining in duodenal biopsies of patients with grade ≥ 2 compared with patients with grade <2 was detected (p=0.0013, Figure 2C).

The numbers of $CD4^+$ expressing IL-17 or Foxp3 was low (Figure 1): CD4 Th17 ranging from 0 to 8 cells per field with a median of 1 (IQR 0.25–4); CD4 T_{reg} median being 4 (IQR 1–6), with a median Th17/T_{reg} ratio of $\frac{1}{2}$ (Table 1A). Severe apoptosis, assessed by TUNEL, was found in 29 patients, Fas positive mononuclear cells in 23, significant TNF alpha expression in 20 and TNF receptor 1 in 26 (Table 1B).

In the 34 patients with pathological GvHD, CD4 Th17 cells numbers ranged between 0 and 7 with a median of 1, whereas CD4-T_{reg} ranged between 0 and 11 with a median of 5 (Table 1A). A Th17/T_{reg} ratio<1 correlated both with the clinical (sensitivity 74%, specificity 100%, p=0.001), and pathological grade ≥ 2 GvHD (sensitivity 94%, specificity 64%, p<0.001). Apoptotic epithelial cells were associated with more than 4 Th17 cells per field in only 3 biopsies but with more than 4 T_{reg} cells in 19 out of 34 biopsies. In the 6 biopsies without apoptotic cells, the corresponding figures were 5 and 0, respectively. A similar trend was found for Fas positive mononuclear cells. In the 17 biopsies with Fas negative cells, the corresponding figures were 7 and 1. In 20 biopsies with high TNF alpha expression, none had more than 4 Th17 cells, while 17 had more than 4 T_{reg} cells per field. In 20 biopsies without high TNF expression, the corresponding figures were 8 and 2. As a consequence, a Th17/T_{reg} ratio<1 correlated with the intensity of apoptosis of epithelial cells (quantitative TUNEL assay), with Fas expression in the cellular infiltrate, and interestingly highly correlated with TNF and TNF receptors 1 and 2 expression (p<0.001 for all) (Table 1B).

We next wanted to confirm, in an independent cohort (2^{nd} cohort), results concerning Th17 and T_{reg} cells in human GI GvHD and to search if the intensity of the conditioning regimen (reduced intensity vs. myeloablative) influences the expression ratio of these 2 cell subsets. As summarized in Figure 2 and Table 2 , we found nearly the same results than in the previously studied cohort regarding Th17 and T_{reg} cell density with regard to GvHD occurrence. However, neither Th17 (p=0.9) or T_{reg} cells (p=0.5) numbers, nor Th17/T_{reg} were influenced by the type of conditioning regimen.

While Foxp3 has a central role in T_{reg} development, it is also clear that Foxp3 upregulation occurs with T cell activation. Thus, as an additional control of the specificity of our results, we used CD8/Foxp3 double staining on the two first cohorts. As shown in Figure 3 , $CD8^+$ $Foxp3^+$ cells counts were negligible compared with CD8+ cells in both first and second cohort whatever the GvHD status (median of 1 and 0.5 cells per field for $CD8^+$ $Foxp3^+$, and 30 and 25.5 cells for $CD8^+$, respectively) (Figure 3).

Since recent experimental results in a mouse model suggest that Th17 cells might be more involved in skin GvHD rather than in GI GvHD [4 , 7 –11] , we analyzed data in an additional cohort of 25 patients (3^{rd} cohort) with acute skin rash or chronic lichenoid skin lesions who underwent skin biopsies (Figure 4). As summarized in Table 3 , we found again similar results regarding Th17 and T_{reg} cell ratio with regard to acute GvHD occurrence. Results in chronic GvHD were statistically insignificant with regard to Th17 and T_{reg} cell numbers but showed similar trends with regards to Th17/T_{reg} .

Finally, we assessed the presence of Th17 and T_{reg} subsets in the PBMCs of 31 patients of the second (GI biopsied) and third cohort (skin biopsied). Samples were analyzed from prospectively collected material at a mean of 3 months post transplantation. Five healthy individuals were included as controls. Because some patients were highly lymphopenic after hematopoietic stem cell transplantation, both subsets were detectable in 23 of the 31 patients. Th17 and T_{reg} frequencies were then correlated to the presence of GvHD. Within the 23 patients, 8 did not have sign of GvHD at time of sampling while 12 presented acute and 3 chronic GvHD. The T_{reg} cell percentage was significantly lower in the 12 patients with acute GvHD than in the 8 patients without GvHD (1.6 ± 0.35% versus 0.46 ± 0.1%; p<0.001) (Figure 5A). For the 3 patients with chronic GvHD, the percentage of T_{reg} cells was 1.7 ± 1.0%. However, we found no correlation between Th17 cell percentage and GvHD occurrence (Figure 5B). The Th17/T_{reg} ratio was statistically no different between patients with or without signs of acute GvHD (p=0.14, Figure 5C).

Discussion

In total, 96 patients' biopsies have been analyzed here to assess the potential role of Th17 cells in human GvHD. We first explored a cohort of 40 patients with suspected GvHD of the GI tract. Th17/T_{reg} correlated both with the clinical diagnosis and disease severity as assessed by pathological grade or by the intensity of the alloimmune reaction (apoptosis of epithelial cells, Fas expression, TNF and TNF receptors). We then assessed Th17/T_{reg} ratio in two other independent cohorts; a second cohort of patients with GI biopsies confirmed that Th17/T_{reg} ratio<1 correlated both with the clinical diagnosis and pathological grade irrespective of what the pre-transplant conditioning regimen was. Finally patients with skin GvHD were studied, and results in this last cohort of patients with skin disease confirmed those found in patients with GI-GvHD.

The Th17/T_{reg} ratio we found in this human GvHD study does not support the hypothesis of a reciprocal relationship between Th17-induced pathology and T_{reg} regulatory role proposed for autoimmune diseases [2 , 16] , especially in the GI tract. CD4 Th17 cells have been characterized in situ in human autoimmune diseases including Sjogren's syndrome, Crohn's disease and rheumatoid arthritis in

humans 17 –20 . In this first study of Th17 in human GvHD we found a lower number of CD4 Th17 cells than in our 5 controls of inflammatory active Crohn's disease. This lower percentage of Th17 in GvHD as compared to Crohn's disease should be kept in mind since this latter disease is usually considered as the prototypic Th1-Th17 GI disease 21 . T_{reg} cells numbers we detected in the GvHD biopsies are in agreement with Rieger and coworkers study 22 who found a mean of 5.4 cells/field and few T_{reg} cells around crypts in acute GvHD patients. It is of note however that, contrary to these authors, we did not found increased numbers of T_{reg} in patients without GvHD.

Today all our knowledge on the role of Th17 cells in GvHD comes from experimental mice studies that lead to divergent conclusions. The role of Th17 has been studied in idiopathic pneumonia syndrome and chronic GvHD rodent models 8 –9 . Th17 was most recently studied in experimental acute GvHD. Carlson and coworkers 7 reported that high dose of in vitro differentiated Th17 mediated severe cutaneous and pulmonary lesions. Yi and coworkers 11 showed that IL-17−/− donor T cells had high Th1 differentiation and induced severe acute GvHD damage. This is in accordance with the results of our human study, where a low Th17/T_{reg} ratio also correlated with severe clinical and pathological GvHD, apoptosis intensity and TNF alpha expression. However, a limitation inherent to our human study design is that no sequential biopsies (i.e. in non symptomatic patients, early after transplantation) were performed. Thus recent experimental results of Kapple and coworkers 4 and of Iclozan and colleagues 23 suggesting that Th17 contributes to the early phase of CD4-mediated GvHD cannot be ascertained in the present study. In another recent study Yi and coworkers 10 used either wild-type mice donor CD4+ T cells that lead to a predominantly Th1 cell mediated disease that preferentially mediated GVHD tissue damage in the gut and liver. However, when authors used IFN-γ−/− CD4+ T cells GvHD that enhanced Th2 and Th17 differentiation and exacerbated tissue damage in the lung and skin; absence of both IL-4 and IFN-γ resulted in enhanced Th17 differentiation and preferential, although not exclusive, tissue damage in the skin. The tissue-specific GVHD mediated by Th1, Th2, and Th17 cells was, in part, associated with their tissue-specific migration mediated by differential expression of chemokine receptors. In view of such results and those obtained using Th17-expanded cells that also induced a skin prominent disease, we also analyzed skin biopsies but we did not find evidence for Th17 cells expansion in skin GvHD in humans. It should also be stressed that cyclosporine A use in human to prevent GvHD also inhibit IL-17 secretion 24 and may have altered fate of Th17 cells in human GvHD.

Most recently the relationship between Th17 and T_{reg} has gained a further level of complexity. Indeed, $CD25^{high}$ $Foxp3^{+}$ regulatory T cells might be able to differentiate into IL-17-producing cells 25 –26 . This has also to be considered for GvHD analysis in human. However we never found cells co-expressing IL-17 and Foxp3 in human biopsies.

Although this study represents the first one in human GvHD and is based on the analysis of nearly one hundred biopsies, we acknowledge some limitations. First larger number of patients with chronic GvHD should be studied. Second, a biopsy is only able to depict a situation (photography) at a given time post transplant even if this time correspond to early onset of clinical signs. Analyses we performed on these 2 cell subsets in peripheral blood found no correlation between Th17 cell percentage and GvHD occurrence and the Th17/Treg ratio was not statistically different between patients with or without signs of GvHD. Thus with the limitation inherent to human studies we cannot rule out that early pathogenetic mechanisms in human GvHD involve a prominent role of the Th17 cell subset. This is particularly relevant in view of recent results by Litjens and coworkers 27 who demonstrated in vitro that the repertoire of alloreactive $CD4^{+}$ T cells is biased to a Th17 response with an average 24% of alloreactive $CD154^{+}$ $CD4^{+}$ memory T cells producing IL-17 after polyclonal stimulation. Unexpectedly, in this study mixed cell cultures from HLA-identical donors also generated alloreactive $CD154^{+}$ $CD4^{+}$ T cells and yielded the highest frequency compared with HLA-non identical combinations.

In conclusion Th17 in human GvHD were not associated with evidence of severe tissue damage at disease onset, but in situ quantification of the Th17/T_{reg} ratio was a specific marker of human GvHD.

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Footnotes:

Responsibilities GS designed the study and with PR and AJ analyzed the data and wrote the manuscript; CL and AD performed immunochemistry analyses; PB contributed to immunohistochemistry analyses; EC and CD collected and prepared PBMC; KK and RPL performed flow cytometry and analyzed data; CP and AQ collected and summarized clinical data; MR PR and RPL recruited patients and participated in manuscript discussion; JYM performed statistical analyses and actively participated to the writing of the manuscript.

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Figure 1

Th17 and Treg distribution in gut biopsies of patients with mild and severe GvHD

Th17 and T_{reg} were identified by the expression of IL-17 or Foxp3, respectively in CD4+ cells using 2-colour immunohistochemistry (IL-17 in brown: A, B; Foxp3 in brown C, D; and CD4 in red; A,B,C,D) Th17 cells were more numerous in patients with mild GvHD (arrowhead: A) whereas T_{reg} were present in patients with severe GvHD (arrowhead: D) Counts of double immunostained cells were independently assessed by 2 pathologists (PR and AJ) on an Olympus AX 70 microscope with wide-field eyepiece number 26.5. At 400× magnification, this wide-field eyepiece provided a field size of 0.344 mm^2 .

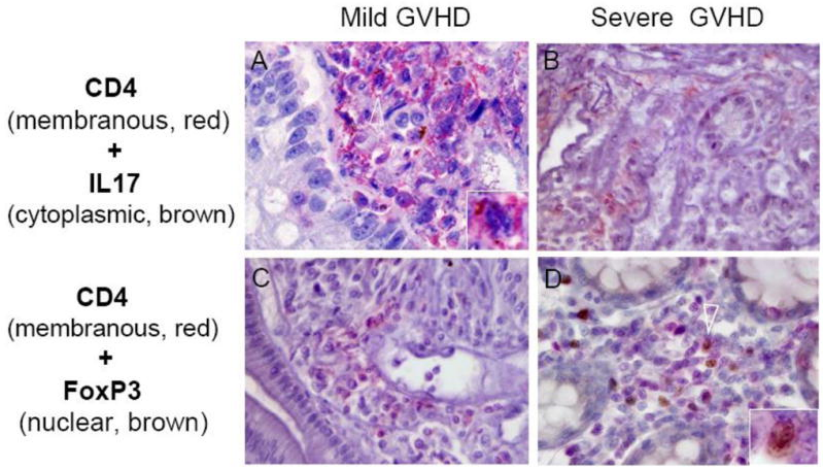


Figure 2

$CD4^+$ counts, $CD4^+$ $Foxp3^+$ and CD4+Foxp3+ cell proportions in duodenal biopsies of patients with grade <2 or ≥ 2 (1^{st} and 2nd cohorts)

A, Absolute number of $CD4^+$ cells per field in the first and second cohorts of duodenal biopsie B, Percentage of $CD4^+$ cells expressing Foxp3 in the same cohort C, Percentage of $CD4^+$ cells expressing IL-17 in the same cohort An increased proportion of $CD4^+$ cells express Foxp3 in duodenal biopsies of patients with grade ≥ 2 compared with patients with grade <2 in the two cohorts A decreased proportion of $CD4^+$ cells express IL-17 in duodenal biopsies of patients with grade ≥ 2 compared with patients with grade <2 in the two cohorts.

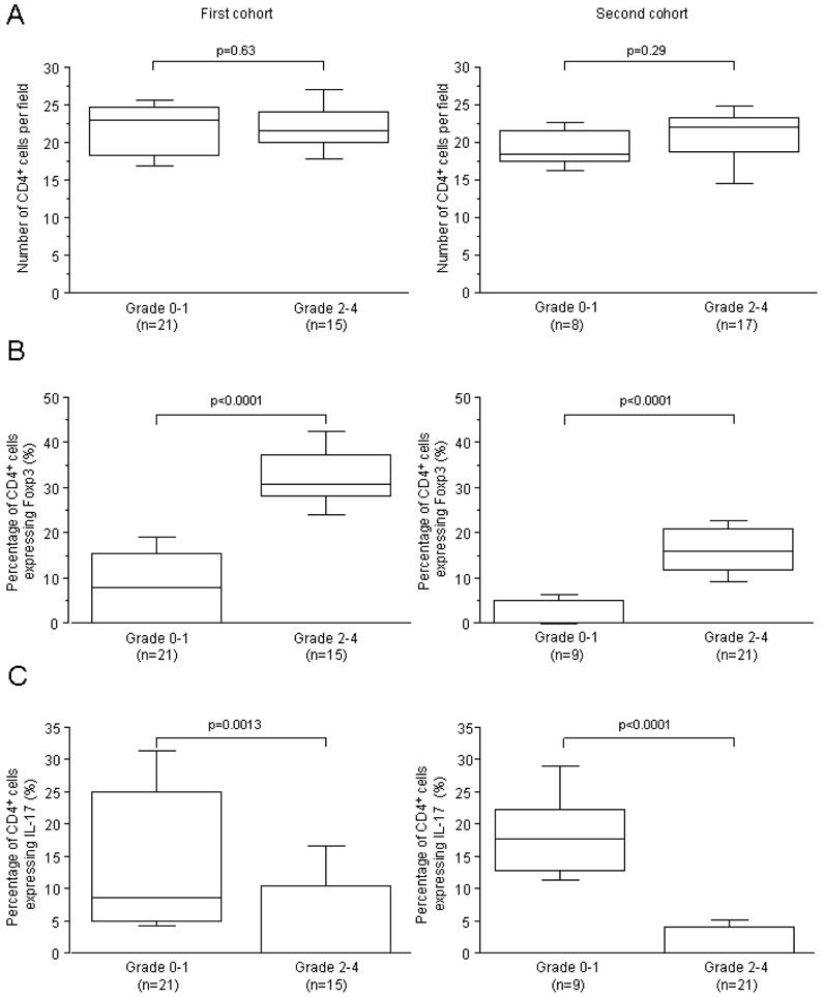


Figure 3

CD8+ and CD8+Foxp3+ cells in duodenal biopsies in the 1st and 2nd cohorts

Number of $CD8^+$ and $CD8^+$ $Foxp3^+$ cells in GvHD patients with grade <2 or ≥ 2. While Foxp3 has a central role in T_{reg} development, it is also clear that Foxp3 upregulation occurs with T cell activation. Thus, as a control of the specificity, CD8/Foxp3 double staining was performed on the two first cohorts. Counts showed that most of the $CD8^+$ cells are not activated in the 2 independent cohorts of patients who underwent duodenal biopsies.

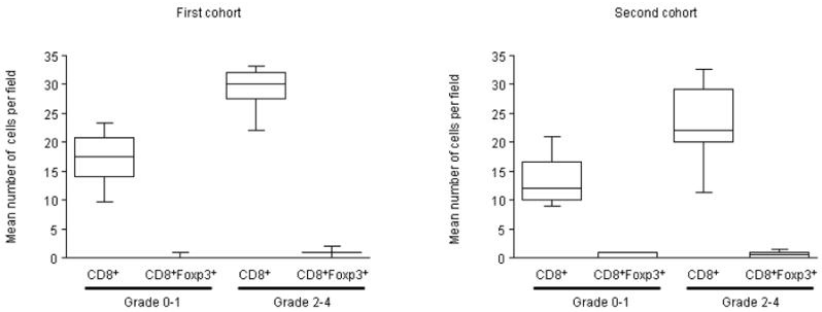


Figure 4

Double immunofluorescent staining with IL-17 and Foxp3 in skin biopsies of patients with acute, chronic and no GvHD

A, Skin biopsies showed infiltrates and apoptotic bodies (arrows) in acute and chronic lichenoid eruption but not in patients without GvHD. B, Overlay of double immunofluorescent staining with IL-17 (red) and Foxp3 (green) antibodies showed Foxp3 expressing cells in skin biopsies of patients with acute and chronic GVHD and IL17-expressing cells in skin biopsies of patients without GVHD. C, Enlargement of the stained cells showed that no of them expressed both IL- 17 and Foxp3.

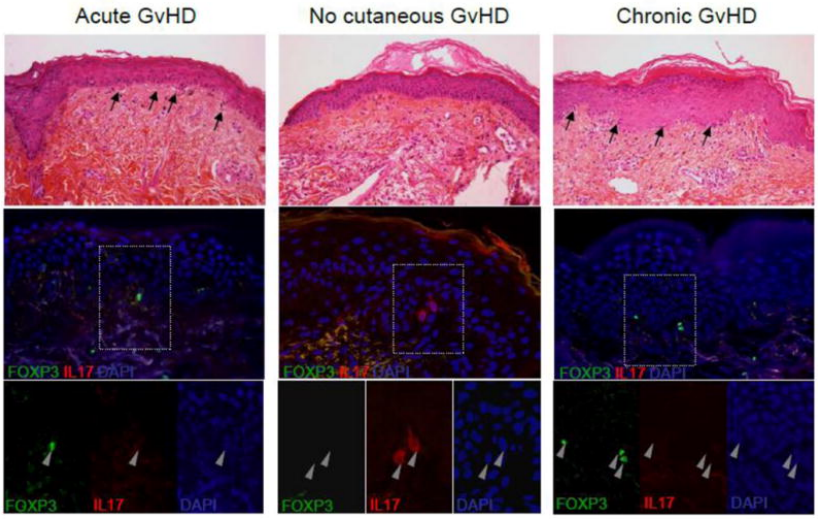


Figure 5

Flow cytometry analyses of Th17, and of T_{regs} in the peripheral blood of 31 patients with acute, chronic or no chronic GvHD

Flow cytometry analyses of Th17, and of T_{regs} in the peripheral blood of 23 patients with acute, chronic or no chronic GvHD PBMCs from healthy controls (n = 5) and patients with GvHD (acute, n=12 and chronic, n=3) or without (n=8) were stained with anti-CD3, anti-CD4, anti-CD25 antibodies followed by intracellular Foxp3 antibody and examined by flow cytometry. Intracellular IL-17 antibody was also examined by flow cytometry after stimulation for 6 hours with PMA and Ionomycine. A, Frequencies of T_{regs} in patients and controls B, Frequencies of Th17 T cells in patients and controls C, Th17/T_{regs} ratio in patients and controls The mean value of each group is represented (—).

Figure 5a

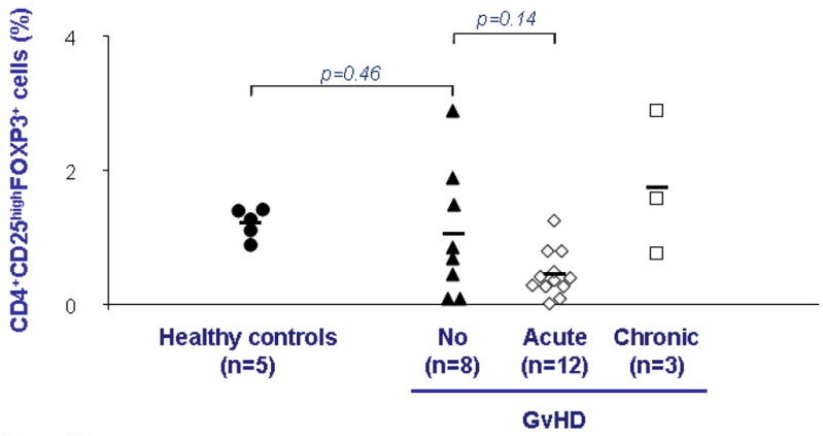


Figure 5b

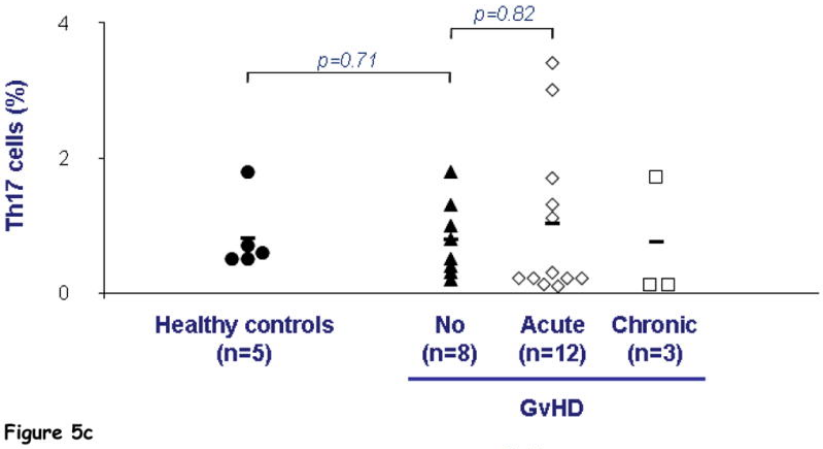


Figure 5c

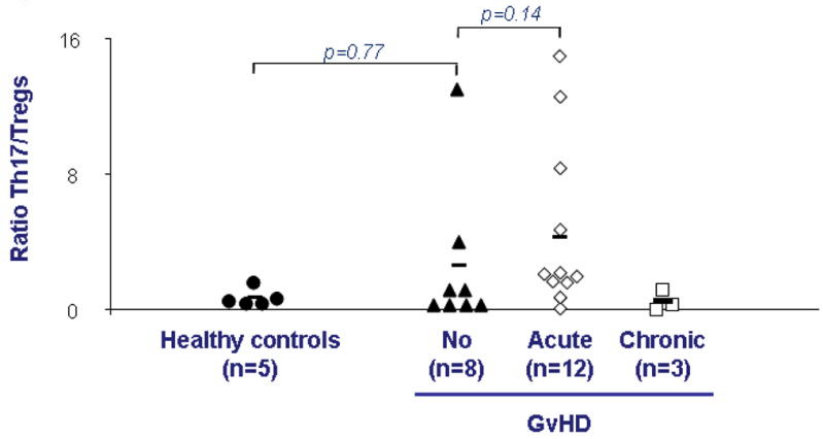


Table 1A

Th17 and T_{reg} numbers in 40 patients with and without clinical or pathological digestive GvHD (1st cohort)

	Total (n=40)	GvHD clinical (final diagnosis)		GvHD pathologically proven			
		No (n=6)	yes (n=34)	No (n=6)	Yes (n=34)	Grade 0–1 (n=22)	Grade 2–4 (n=18)
Th17 median (range)	1.0 (0–8)	5.5 (1–8)	1.0 (0–7)	5.5 (4–8)	1.0 (0–7)	2.0 (0–8)	0.5 (0–4)
Treg median (range)	4.0 (0–11)	0.0 (0–1)	5.0 (0–11)	0.0 (0–3)	5.0 (0–11)	2.0 (0–6)	6.5 (0–11)
Th17/Treg *median (range)	0.5 (0.05–16)	9.0 (2–16)	0.4 (0.05–10)	10 (2.3–16)	0.4 (0.05–6)	1.5 (0.13–16)	0.1 (0.05–4)
Th17/Treg <1 n (%)	25 (63%)	0 (0%)	25 (74%)	0 (0%)	25 (74%)	8 (36%)	17 (94%)

Table 1B

Correlations of Th17 and T_{reg} numbers, and Th17/T_{reg} ratio with other GvHD pathological markers (1st cohort)

	Th17 >4	Treg >4	Th17/Treg < 1	p[1]
Apoptotic epithelial cells (TUNEL)				
No (n=6)	5	0	0	0.001
Yes (n=34)	3	19	25	
More than 5 per field (n=29)	0	10	15	0.03
Less than 5 per field (n=11)	8	9	10	
Apoptotic cells within the infiltrate (TUNEL)				
No (n=19)	8	3	7	0.03
Yes (n=21)	0	16	18	
Fas expression within the cellular infiltrate				
No (n=17)	7	1	6	0.003
Yes (n=23)	1	18	19	
More than 5 cells per field (n=8)	0	7	1	0.2
Less than 5 cells per field (n=32)	8	12	18	
TNF receptor 1				
No (n=14)	8	0	3	<0.001
Yes (n=26)	0	19	22	
TNF receptor 2				
No (15)	8	0	3	<0.001
Yes (n=25)	0	19	22	
TNF				
Less than 20 cells per field (n=20)	8	2	7	<0.001
More than 20 cells per field (n=20)	0	17	18	

[1] Fischer's exact test for the association of Th17/Treg <1 with the other pathological parameters

Table 2

Th17 and T_{reg} numbers in 30 patients with and without pathological digestive GvHD, according to conditioning regimen (2nd cohort)

	Acute GvHD MAC	Acute GvHD RIC	No GvHD	GvHD after MAC vs. no GvHD	GvHD after RIC vs. no GvHD
	n=11	**n= 10**	**n= 9**	**p (Mann Whitney)**	**p (Mann Whitney)**
Th17 median (range)	0 (0–1)	0 (0–2)	3 (2–7)	.0001	.0002
Treg median (range)	3 (1–7)	3.5 (2–6)	0 (0–1)	.0002	.0002
Th17/Treg median (range)	0.25 (0.14–0.50)	0.21 (0.10–0.50)	4.00 (3.00–10.00)	.0001	.0002
Th17/Treg <1 * n (%)	11 (100%)	10 (100%)	0 (0%)	<.0001*	<.0001*

* Fischer exact test

Table 3

Th17 and T_{reg} numbers in 26 patients with and without pathological cutaneous GvHD (3rd cohort)

	Acute GvHD	Chronic GvHD	No GvHD	Acute GvHD vs. no GvHD	Chronic GvHD vs. no GvHD
	n= 10	**n= 5**	**n= 11**	**p (Mann Whitney)**	**p (Mann Whitney)**
Th17 median (range)	0(0–2)	1 (1–2)	2 (1–5)	.0004	.062
Treg median (range)	2.5 (2–5)	3 (2–3)	1 (0–2)	.0003	.25
Th17/Treg median (range)	0.25 (0.17–0.40)	0.50 (0.33–1.00)	2.00 (1.50–4.00)	.0001	.001
Th17/Treg <1 * n (%)	10 (100%)	4 (80%)	0 (0%)	<.0001*	<.003*

* Fischer exact test