Gut Inflammation in axial Spondyloarthritis patients is characterized by a marked Type 17 skewed mucosal Innate-like T cell signature

Supplementary Materials and Methods

Ileocolonoscopy

For histopathological assessment, 6 biopsies from ileum and 8 from colon (2 per colon segment: ascendens, transversum, descendens and sigmoid) were collected in formaldehyde. For flow cytometric analysis, the same amount of biopsies were collected in RPMI1640 medium (GibcoTM, 72400-054), containing 10% fetal calf serum (FCS), 1% Penicillin-Streptomycin (GibcoTM, 15140-122), 1% Sodium Pyruvate (GibcoTM, 11360-070) and 1% non-essential amino acids (GibcoTM, 11140050).

Isolation IEL and LPL

Cell isolation was performed immediately following collection of gut biopsies. Biopsies were washed in RPMI1640 medium and subsequently in PBS (Gibco™, 10010-056). The weight of the biopsies was recorded, in order to normalize absolute cell counts per mg of tissue. On average, we obtained 40-60mg tissue from 6-8 biopsies. First, biopsies were incubated for 15 minutes at 37°C, in a HBSS (Gibco™, 14175-095) solution containing 2mM EDTA, 0.1% Gentamicin (Gibco™, 15750-037) and 1% Penicillin-Streptomycin (Gibco™, 15140-122), with continuous stirring at 300rpm. Following incubation, the solution was passed through a 40µm filter, PBS was added and centrifugation was done for 7 minutes at 393g, 4°C. Cell pellets were collected, and resuspended in FACS buffer (PBS containing 2% FCS), yielding the intraepithelial cells. Remaining gut material was further digested in RPMI160 containing 1mg/ml of collagenase IV (Sigma-Aldrich, C5138) and collagenase VIII (Sigma-Aldrich, C2139), for 30 minutes at 37°C, with continuous stirring at 500rpm. Next, the solution was passed through a 40µm filter, washed and resuspended in FACS buffer as before, yielding the lamina propria cells. Cell counting was done using a Bürker counting chamber.

Intracellular flow cytometry

For transcription factor staining, single-cell suspensions from PBMC, IEL and LPL were stained directly *ex vivo*.

For the functional cytokine assays, single-cell suspensions from LPL cells were re-stimulated *ex vivo* for 4 hours with PMA (25ng/ml, Sigma-Aldrich, P1585), Calcium-ionomycin (1µg/ml, Sigma-Aldrich, I0634-1MG) and Brefeldin A (1µg/ml, BD, 555029) or with only Brefeldin A as a negative control, prior to cytokine staining.

FACS staining was performed at 4°C, as described before (1). BD Horizon™ Fixable Viability Stain 700 (564997) or Fixable Viability Stain 520 (564407), and human FcR Blocking Reagent (Miltenyi, 130-059-901) were used to respectively exclude dead cells and aspecific FcR binding of antibodies. FACS staining was performed using fluorochrome conjugated antibodies, as shown in Suppl. Table 4 and 5. For intracellular transcription factor staining, eBioscience™ FoxP3/Transcription Factor Staining Buffer Set (00-5523-00) was used according to manufacturer's instructions. For intracellular cytokine staining, BD Cytofix/Cytoperm™ Plus (555028) was used according to manufacturer's instructions. Samples

were filtered before acquisition using a pre-separation filter of 30µm (Miltenyi, 130-041-407). Acquisition was performed on a BD FACSAria™ III flow cytometer and data were analysed using FlowJo™ v10.6.2 software. The gating strategy is depicted in Suppl. Fig. 1. Absolute cell counts are expressed per mg tissue for intestinal samples, and per ml of blood for PBMC.

FlowSOM

Preprocessing

Compensation, transformation and pre-gating was done with FlowJo™ v10.6.2 software, exporting fcs files containing only the T cells. These where then imported in R, where margin events and low quality events were removed with PeacoQC (2). The fcs files were then, based on the manual gating, split into two subsets of interest: the innate-like T cells and the conventional T cells, as they will be separately clustered in the next step.

Clustering

FlowSOM (3), a two-level clustering algorithm, was used to cluster the T cells. Two FlowSOM models were computed, every time using an aggregate of maximum 10000 cells per fcs file. Each model had 49 clusters and 10 meta-clusters. IEL, LPL and PBMC fractions were combined, but the data was split into the innate-like T cells (917,242 cells; TCRVβ11, TCRγδ, TCRVα7.2, 6B11, CD3) and the conventional T cells (1,441,643 cells; TCRVβ11, TCRVα7.2, CD161). Finally, abundances of all clusters were obtained from these FlowSOM models, i.e. percentages of T cells, as visualized by the node sizes in the FlowSOM trees.

Transcription factor quantification

To characterize the T cell subsets from the FlowSOM clustering in more detail, we looked at two transcription factors: Tbet and RORγt. The deGate function of the flowDensity package (4) was used to estimate the optimal threshold in the density distributions of the transcription factors. To optimize the deGate algorithm, we tried out twelve different parameter sets and selected the best one for each FlowSOM object and transcription factor combination based on the approximation of the manual gate in FlowJo. Per FlowSOM metacluster, the percentages of cells that are positive for each transcription factor were calculated.

Statistics

Finally, statistical comparisons were conducted to investigate differences between healthy controls and spondylarthritis patients with and without histological events of gut inflammation. We performed these tests per tissue fraction per FlowSOM object. This resulted in 10 groups for the statistical analysis: five for innate-like T cells (PBMC, IEL_colon, LPL_colon, IEL_ileum, LPL_ileum), and five for conventional T cells (PBMC, IEL_colon, LPL_colon, IEL_ileum, LPL_ileum). Per group, two contrasts were investigated: healthy controls versus SpA patients and SpA patients without gut inflammation versus with inflammation. Median values for the features (e.g. percentage of RORγt positive cells) were calculated per cluster and visualized on the FlowSOM trees. Log fold changes comparing the two conditions were calculated, corrected (if percentages were 0 in both cases, a log fold change is set to 0; +/- Infinity is substituted with the -/+ max log fold value) and also visualized on the FlowSOM tree.

Supplementary Tables

Supplementary Table 1. Patient characteristics of nr-axSpA patients (with and without gut inflammation) and healthy controls, used for flow cytometric immunophenotyping of innate-like T cells and conventional T cells on peripheral blood mononuclear cells (PBMC), intra-epithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL). Shown as either n per group for dichotomous parameters, or as mean ± 95% confidence interval for continuous variables. An overview of the amount of samples collected is given per tissue (eg. no ileum samples in case the ileum could not be accessed during the colonoscopy, no intraepithelial lymphocytes in case the cell yield was too low further analysis). Due to low cell yield in certain samples and given the technical complexity, not all analyses could be performed on all samples. HLA: human leucocyte antigen; ASDAS: Ankylosing Spondylitis Disease Activity Score; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; BASFI: Bath Ankylosing Spondylitis Functional Index; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; NSAID: non-steroidal anti-inflammatory drug.

*NSAID index: the NSAID intake score, recommended by ASAS, quantifies the cumulative dose intake of NSAIDs on a scale of 0–100 over the analysed time period, where 100 represents the maximum approved dose of the NSAID and 0 represents no intake at al. The analysed time period is 30 days before the colonoscopy.

	SpA patients	SpA with gut inflammation	SpA without gut inflammation	Healthy control
n	25	11	14	15
Origin: Caucasian/not Caucasian	23/2	10/1	13/1	15/0
Female/Male	12/13	3/8	9/5	12/3
Age (years)	31.6 (28.1 - 35.1)	33.0 (27.6 - 38.4)	30.5 (25.2 - 35.8)	49.4 (43.0 - 55.8)
Gut inflammation				
Ileum/colon/both	6/3/2	6/3/2	na	na
Acute/chronic	8/3	8/3	na	na
Disease duration (years)	2.6 (0.9 - 4.2)	3.8 (0.1 - 7.6)	1.6 (0.3 - 3.0)	
HLA-B27: +/-	19/6	10/1	9/5	
ASDAS	2.9 (2.4 - 3.4)	2.7 (1.9 - 3.6)	3.1 (2.5 - 3.8)	
BASDAI	5.0 (4.0 -5.9)	4.5 (2.7 - 6.4)	5.3 (4.2 - 6.4)	
BASFI	3.3 (1.1 - 5.6)	3.9 (2.7 - 5.0)	4.3 (2.9 - 5.7)	
CRP (mg/dL)	9.0 (4.0 - 14.0)	6.6 (0.2 - 13.0)	10.8 (2.9 - 18.6)	
ESR (mm/h)	16.2 (10.2 - 22.3)	18.8 (6.1 - 31.6)	14.2 (8.3 - 20.2)	
NSAID index*	26.85 (14.60 - 39.11)	27.15 (3.77 - 50.52)	26.63 (10.97 - 42.29)	
Samples collected				
PBMC		11	14	14
IEL ileum		10	13	9
IEL colon		11	13	9
LPL ileum		10	13	10
LPL colon		11	13	12

Supplementary Table 2. Patient characteristics of axSpA patients (with and without gut inflammation), used for serum IL-17A measurement by Luminex. Shown as either n per group for dichotomous parameters, or as mean ± 95% confidence interval for continuous variables. HLA: human leucocyte antigen; ASDAS: Ankylosing Spondylitis Disease Activity Score; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; CRP: C-reactive protein; NSAID: non-steroidal anti-inflammatory drug.

*NSAID index: the NSAID intake score, recommended by ASAS, quantifies the cumulative dose intake of NSAIDs on a scale of 0–100 over the analysed time period, where 100 represents the maximum approved dose of the NSAID and 0 represents no intake at al. The analysed time period is 3 months before the colonoscopy.

	SpA patients	SpA with gut inflammation	SpA without gut inflammation
n	97	43	54
Female/Male	50/47	21/22	29/25
Age (years)	35.0 (33.0 - 37.0)	33.3 (30.2 - 36.4)	36.4 (33.8 - 38.9)
HLA-B27: +/-	75/22	33/10	42/12
ASDAS	2.7 (2.5 - 2.9)	2.8 (2.4 - 3.1)	2.6 (2.3 - 2.9)
BASDAI	4.5 (4.1 - 4.9)	4.6 (3.9 - 5.2)	4.4 (3.9 - 5.0)
CRP (mg/dL)	7.4 (5.0 - 9.8)	7.9 (4.4 - 11.5)	7.0 (3.7 - 10.0)
NSAID index*	51.8 (43.8 - 59.8)	46.7 (37.1 - 56.3)	56.0 (43.5 - 68.5)

Supplementary Table 3. Patient characteristics from a subset of nr-axSpA patients, which were followed-up longitudinally. Collection of PBMC for flow cytometric immunophenotyping was done at baseline (prior to treatment, W0), after 4 weeks of NSAID treatment (W4) and when applicable, after 6 months of anti-TNF therapy (W28). Shown as either n per group for dichotomous parameters, or as mean ± 95% confidence interval for continuous variables. HLA: human leucocyte antigen; ASDAS: Ankylosing Spondylitis Disease Activity Score; BASDAI: Bath Ankylosing Spondylitis Disease Activity Index; BASFI: Bath Ankylosing Spondylitis Functional Index; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate.

	SpA patients - baseline (W0)	SpA patients - NSAID (W4)	SpA patients - antiTNF (W28)
n	12	12	7
Origin: Caucasian/not Caucasian	11/1	11/1	6/1
Female/Male	7/5	7/5	4/3
Age (years)	31.2 (26.0 - 36.3)	31.2 (26.0 - 36.3)	29.6 (22.0 - 37.2)
Gut inflammation			
Yes/no	4/8	4/8	2/5
Ileum/colon/both	2/2/0	2/2/0	0/2/0
Acute/chronic	3/1	3/1	1/1
Disease duration (years)	0.8 (0.6 - 1.1)	0.8 (0.6 - 1.1)	0.9 (0.4 - 1.5)
HLA-B27: +/-	8/4	8/4	4/3
ASDAS	3.3 (2.7 - 3.9)	3.3 (2.7 - 3.9)	3.3 (2.5 - 4.1)
BASDAI	5.1 (3.8 - 6.5)	5.1 (3.8 - 6.5)	5.3 (2.9 - 7.7)
BASFI	3.9 (2.1 - 5.6)	3.9 (2.1 - 5.6)	4.7 (2.3 - 7.2)
CRP (mg/dL)	11.7 (1.9 - 21.5)	11.7 (1.9 - 21.5)	11.6 (0.0 - 23.8)
ESR (mm/h)	16.8 (10.4 - 23.2)	16.8 (10.4 - 23.2)	16.6 (6.6 - 26.5)

Supplementary Table 4. Antibodies used for intracellular transcription factor flow cytometry staining.

Fluorochrome	**Marker**	**Supplier**	**Cat no**	**Clone**
FITC	TCRVβ11	Beckman Coulter	IM1586	C21
PERCP-Cy5.5 (eFluor 710)	TCRγδ	eBioscience	46-9959-42	B1.1
APC (eFluor 660)	Tbet	eBioscience	50-5825-82	eBio4B10
APC-Cy7 (APC-eFluor 780)	CD3	eBioscience	47-0038-42	UCHT1
PE-Cy7	CD161	eBioscience	25-1619-41	HP-3G10
Alexa Fluor 700	FVS700	BD	L34967	NA
BV605	TCRVα7.2	Biolegend	351720	3C10
BV421	RORγt	BD	563282	Q21-559
BV510	TCRVα24	BD	563267	6B11

Supplementary Table 5. Antibodies used for intracellular cytokine flow cytometry staining.

Fluorochrome	Marker	Supplier	Cat no	Clone
FITC	FVS520	BD	564407	NA
PE	TCRVβ11	Beckman Coulter	IM2290	C21
PERCP-Cy5.5 (eFluor 710)	IL-17A	eBioscience	45-7179-42	eBio64DEC17
APC (eFluor 660)	CD161	BD	550968	DX12
APC-Cy7 (APC-eFluor 780)	CD3	eBioscience	47-0038-42	UCHT1
PE-Cy7	IL-22	eBioscience	25-7229-42	22URTI
BV605	TCRVα7.2	Biolegend	351720	3C10
BV421	TCRγδ	BD	562560	B1
BV510	TCRVα24	BD	563267	6B11

Supplementary Figure Legends

Supplementary Figure 1. Gating strategy for immune profiling by intracellular flow cytometry. Representative flow cytometry plots are shown for lamina propria lymphoyctes (LPL). γδ-T cells are defined as CD3+ and TCRγδ+. MAIT cells are defined as CD3+, NOT-γδ-T cells, CD161+ and TCRVα7.2+. iNKT cells are defined as CD3+, NOT-γδ-T cells, NOT-MAIT cells, TCRVβ11+ and TCRVα24 (clone 6B11)+. Conventional T cells (Tconv) are defined as CD3+, NOT-γδ-T cells, NOT-MAIT cells and NOT-iNKT cells. Conventional T cells are subdivided in CD161+ and CD161- cells. Intracellular staining is shown for γδ-T cells. Panel 1: intracellular staining on transcription factors RORγt and Tbet. Panel 2: intracellular cytokine staining after stimulation with PMA and CaI, for IL-17A and IL-22.

Supplementary Figure 2. FMO controls for RORγt and IL-17A staining, shown for γδ-T cells in lamina propria lymphoyctes (LPL).

Supplementary Figure 3. Absolute cell counts of lymphocytes, innate-like T cells and conventional T cells. A. Intra-epithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) from ileum and colon of healthy controls and SpA patients without and with gut inflammation. Absolute cell counts per mg of gut tissue. **B.** PBMC of healthy controls and SpA patients. Absolute cell counts per ml of blood. All graphs show mean ± SEM. IEL: intraepithelial lymphocytes; LPL: lamina propria lymphocytes.

Supplementary Figure 4. γδ-hi cells in PBMC in relation to clinical parameters in SpA patients. A. Correlation of the proportion of γδ-hi cells in PBMC of SpA patients with Bath Ankylosing Spondylitis Disease Activity Index (BASDAI), C-reactive protein (CRP, mg/dl) and erythrocyte sedimentation rate (ESR, mm/h) (Pearson correlation coefficient).

Supplementary Figure 5. Absolute cell counts of RORγt+ γδ-T cells (including the γδ-hi and γδ-int subsets), MAIT cells, CD161+ conventional T cells and CD161- conventional T cells. **A.** PBMC of healthy controls and SpA patients. Absolute cell counts per ml of blood. **B.** Intra-epithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) from ileum and colon of healthy controls and SpA patients without and with gut inflammation. Absolute cell counts per mg of gut tissue. All graphs show mean ± SEM, *P<0.05, **P<0.01. IEL: intraepithelial lymphocytes; LPL: lamina propria lymphocytes.

Supplementary Figure 6. RORγt expression is higher in intestinal innate-like T cells compared to conventional T cells. Percentage of RORγt+ cells in intra-epithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) from ileum and colon, comparing γδ-T cells (including the γδ-hi and γδ-int

subsets), MAIT cells, CD161+ conventional T cells and CD161- conventional T cells. **A.** SpA patients with gut inflammation. **B.** SpA patients without gut inflammation. **C.** Healthy controls. All graphs show mean ± SEM, *P<0.05, **P<0.01.

Supplementary Figure 7. Intracellular IL-22 measurement in LPL. Representative flow cytometry plots of LPL, for functional assay with measurement of intracellular IL-22 in γδ-T cells, MAIT cells and conventional T cells, comparing SpA patients without and with gut inflammation (n=2/group). LPL: lamina propria lymphocytes.

Supplementary Figure 8. Absolute cell counts of Tbet+ γδ-T cells (including the γδ-hi and γδ-int subsets), MAIT cells, CD161+ conventional T cells and CD161- conventional T cells. **A.** PBMC of healthy controls and SpA patients. Absolute cell counts per ml of blood. **B.** Intra-epithelial lymphocytes (IEL) and lamina propria lymphocytes (LPL) from ileum and colon of healthy controls and SpA patients without and with gut inflammation. Absolute cell counts per mg of gut tissue. All graphs show mean ± SEM, *P<0.05. IEL: intraepithelial lymphocytes; LPL: lamina propria lymphocytes.

Supplementary Figure 9. FlowSOM analysis confirms loss of Tbet in SpA patients with gut inflammation. FlowSOM analysis of innate-like T cells **(A)** and conventional T cells **(B)**, showing Tbet expression in lamina propria ileum. Left: mean expression of Tbet in SpA patients without gut inflammation and SpA patients with gut inflammation is shown in white-grey scale. The size of the nodes represents the relative abundance of each cluster. Right: log fold change, comparing SpA patients with and without gut inflammation (red = more in SpA inflamed, blue = more in SpA non-inflamed). Background colors represent the identified cell populations. LPL: lamina propria lymphocytes.

Supplementary Figure 10. TNF inhibition completely restores γδ-hi cell proportion and RORγt levels in peripheral blood. A. Proportion of γδ-hi T cells (left) and percentage of T cells (right) in SpA patients at W0, W4 and W28. **B.** Percentage of RORγt+ γδ-T cells (including the γδ-hi and γδ-int subsets), MAIT cells, CD161+ conventional T cells and CD161- conventional T cells, in SpA patients at W0, W4 and W28. Connecting lines in a separate color per patient indicate samples pre- and post-treatment to follow individual responses. *P<0.05, **P<0.01.

Supplementary Figure 11. Percentage of T cells in PBMC of healthy controls and SpA patients. Mean ± SEM, *P<0.05, **P<0.01.

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