# Specific induction of the unique GPR15 expression in heterogeneous blood lymphocytes by tobacco smoking

Mario Bauer[a*], Jörg Hackermüller[b], Jana Schor[b], Virginie Dubourg[b], Stephan Schreiber[b], Beate Fink[a], Arkadiusz Pierzchalski[a,c], Gunda Herberth[a,c]

[a]Department of Environmental Immunology, Helmholtz Centre for Environmental Research GmbH - UFZ, Leipzig, 04318, Germany.

[b]Young Investigators Group Bioinformatics and Transcriptomics, Helmholtz Centre for Environmental Research-UFZ, Leipzig, 04318, Germany.

[c]These authors contributed equally to this work

*Corresponding author information:

Street address: Helmholtz Centre for Environmental Research GmbH - UFZ

Permoserstraße 15 / 04318 Leipzig / Germany

Telephone number: +49 341 235 1552

FAX: +49 341 235 1787

e-mail: mario.bauer@ufz.de

# Specific induction of the unique GPR15 expression in heterogeneous blood lymphocytes by tobacco smoking


## Abstract

**Purpose:** In the peripheral blood, it has been shown that smoking is, to date, the only specific condition leading to an increase in GPR15+ T cells. We therefore aimed to characterize GPR15-expressing blood T cells in more detail.

**Materials and Methods:** The whole transcriptome by RNAseq as a proxy for protein expression was analyzed in GPR15+ and GPR15- T cells. A deep immuno-phenotyping was conducted for identification of T cell subtypes.

**Results:** The expression of GPR15 seemed to be unique, not concomitantly accompanied with expression of another protein. According to different T cell subtypes there is no single cell type prominently represented in GPR15+ T cells. The individually different proportions of GPR15+ cells among each GPR15-expressing T cell subtypes in blood were strongly associated with chronic smoking. Indeed, the frequency of GPR15+ T cell subtypes can be effectively used as a highly convincing biomarker for tobacco smoking.

**Conclusions:** While the chronic smoking-induced enrichment of GPR15+ T cells in blood might indicate a systemic inflammation, by the widespread presence in different T cell subtypes, GPR15 could feature a general impact on maintaining the systemic homeostasis to putatively prevent harm from smoking.




Keywords: GPR15, MAIT, Th17, Treg, smoking

## Introduction

The recent identification of the chemoattractant ligand GPR15L for the G protein-linked receptor GPR15-expressing T cells suggested an interfering role of GPR15-GPR15L interaction in mucosal and cutaneous inflammation (Ocon et al. 2017). The GPR15L, encoded by *C10orf99*, was found to be an upregulated marker gene for psoriatic pathogenesis in skin (Guo et al. 2014). However, in colon the constitutively expressed GPR15L was only modestly impaired be inflammation or the presence of microbiota.

In human lymphocytes the GPR15 receptor serves as a chemokine receptor for the immunodeficiency virus type 1 and 2 (Deng et al. 1997). In mice, it mediates the homing of lymphocytes to the sites of inflammation in the large intestine (Kim et al. 2013) and of fetal dendritic epidermal T cells (DETCs), overexpressing GPR15, from the thymus to the skin where the GPR15 expression is rapidly downregulated on skin resident DETCs (Lahl et al. 2014). Although the lymphocyte recruitment maintains intestinal immune homeostasis, it also contributes to inflammation (Nguyen et al. 2015). With the presence of GPR15+ regulatory T cells in mice colitis and their absence in human colitis, indeed, there are serious differences between human and mice (Fischer et al. 2015). This hampers the extrapolation of findings from mice to human. Nevertheless, while GPR15+ T cells are enriched in inflamed tissue, such as colon, their frequency in blood remains unchanged. Similarly, gene expression of *GPR15* was not changed in blood of subjects with psoriatric skin compared to healthy subjects (Coda et al. 2012). In contrast to above mentioned chronic systemic inflammatory disorders, chronic smoking is to date the only strong inducer of GPR15+ lymphoid cells in blood (Bauer et al. 2015). The adaptive immunological process of GPR15 activation in lymphoid cells has not yet been resolved. Their excess in blood was not evoked by a suggested smoking-induced disturbed homeostasis of the lung (Bauer et al. 2017). Smoking-induced expression of the *GPR15* gene in blood was recently assumed to indicate its potential role in chronic inflammatory pathologies (Koks et al. 2015). Because of both the strong association between epigenetic hypomethylation at CpG site cg19859270 (annotated to *GPR15*) with cellular GPR15 protein expression (Bauer et al. 2015) and linear correlation between duration and degree of smoking and time since cessation with degree of hypomethylation at cg19859270

(Ambatipudi et al. 2016), moreover at protein level there are strong evidences for the number of GPR15-expressing T cells in blood as a high-sensitive and high-specific biomarker for the degree of systemic disturbed homeostasis by chronic tobacco smoking.

With respect to the recently emerged therapeutic interest in GPR15L-GPR15 interaction for management of chronic diseases, such as autoimmune disorders, the present study aimed at characterization of GPR15+ T cells in blood in more detail. We show that the GPR15 is a unique surface receptor which is not accompanied concomitantly by another cellular protein and not restricted to a specific T cell subtype. Additionally we show the different impact on T cell subtypes of chronic smoking as, to date, the only specific inducer of an excess of GPR15+ T cells in blood.

**Clinical Significance**

- The physiological role of GPR15 remains elusive.
- Proportion of GPR15-expressing T cells in peripheral blood is an unspecific indicator for pathological processes, such as inflammatory bowel disease, but is a highly specific and sensitive biomarker for chronic tobacco smoking.
- Smoking behavior should be considered for suggested therapeutic intervention of the GPR15-GPR15L interaction in autoimmune diseases.

**Materials and Methods**

*Subjects*

Pseudonymous blood samples from volunteers (22 non-smokers and 23 chronic active smokers) were obtained from the blood bank at the University of Leipzig (Figure 1). Smoking behaviour (yes or no), age and gender were recorded via questionnaires. Smoking behavior was rechecked by cotinine level in plasma using the Cotinine direct ELISA Kit according to manufacturer's instruction (DRG Instruments GmbH, Marburg, Germany) Cotinine level exceeding the sensitivity level of the assay (1 ng/ml) was considered as smoker. All participants gave their written informed consent. The study received approval

by the Ethics Committee of the University of Leipzig (reference number 079-15-09032015).

*Analysis of surface proteins*

GPR15 surface expression concomitant with markers of differentiation on lymphocytes was analysed by flow cytometry. Briefly, 100 µl of blood specimen was incubated with mouse-anti-human-GPR15 antibody (1:500; R&D Systems, Wiesbaden-Nordenstadt, Germany) supplemented with 5% goat serum for 1 h. After washing in PBS/1% fetal calf serum (FCS) the GPR15 was stained with R-phycoerythrin-labelled goat-anti-mouse IgG2b (1:500, 1 h; Biozol, Eching, Germany) following an additional wash step. Thereafter, cells were incubated with 5% mouse serum for 30 min following a double-staining step for leucocyte differentiation markers (1 h). T cell subtypes were analysed as follows, T-helper subsets (T helper (CXCR5-), follicular T helper [Tfh], CXCR5+): CD3-FITC, CD4-BV510, CXCR5-PerCP-Cy5.5, CCR4-APC-Vio770, CCR6-PC7, CCR10-APC, CXCR3-BV421; Treg (nTreg (CD3+CD4+CD127lowCD25+), active nTreg (nTregCD39+), Th3 (CD3+CD4+CD25-LAP+)): CD4-APC-Cy7, CD3-PerCP-Cy5.5, CD127-PacidicBlue, CD25-PC7, CD39-FITC; Treg (Tr1): CD4-APC-eF780, CD3-PerCP, CD45RA-PacBlue, CD25-PC7, LAG-3-PerCP-Cy5.5, CD49b-APC-; naïve (CD45RA+CD27+)/central memory (CD45RA-CD27+)/effector memory (CD45RA-CD27-)/effector (CD45RA+CD27-): CD4-BV510, CD45RA-FITC, CD27-PerCP-Cy5.5; MAIT (CD3+,CD4-,CD8+,CD161+,TCR-Vα7.2+): CD3-PerCP-Cy5.5, CD8-APC-eF780, CD4-PC7, CD161-BV421, TCR-Vα7.2-APC. At the end, erythrocytes were lysed in FACS Lysing solution (BD Bioscience, Heidelberg, Germany) according to manufacturer's instruction immediately before measurement. All measurements were performed on a FACS Canto II and analyzed with the BD FACS DIVA software (version 8.0.1, BD Biosciences, Heidelberg, Germany).

*Lymphocyte separation*

Peripheral Blood Mononuclear Cells (PBMCs) were obtained by density-gradient centrifugation using Ficoll-Paque (GE Healthcare, Solingen, Germany). GPR15+CD3+ and GPR15-CD3+ T cells were separated by flow cytometric cell sorting after antibody staining of PBMCs for CD3 and GPR15 surface proteins as indicated above. Flow-cytometric cell sorting was performed at the laboratory of cytometry of the Core Facility at the University of Leipzig.

*Gene expression by quantitative PCR*

For semi-quantitative PCR (qPCR), total RNA of separated leucocytes was prepared by using peqGold RNA Pure (peqlab, Erlangen, Germany) according to manufacturer's instructions. The cDNA synthesis was carried out with 1 µg of RNA by using ImProm-II™ Reverse Transcription System (Promega, Mannheim, Germany). Intron-spanning primers were designed and UPL probes were selected by the Universal Probe Library Assay Design Center (http://qpcr.probefinder.com/organism.jsp) (Table S4). Gene expression of 38 randomly selected genes was measured using the 96.96 Dynamic Array or FLEXsix Integrated Fluidic Circuits (IFCs) (Fluidigm, San Francisco, CA, USA). Gene expression values were determined by using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001) with GAPD and GUSB as reference genes and normalized to the lowest measured value.

*Transcriptome-sequencing*

Total RNA was extracted as described previously (Hackermuller et al. 2014) using miRNeasy Mini Kit® in combination with QIAcube® (QIAGEN GmbH, Hilden, Germany) and TURBO DNA-free™ Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). The RNA integrity for each sample was controlled with the RNA 6000 Nano Assay and the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). All samples included in the experiments had RIN > 8. Ribosomal RNAs were removed from

total RNA using the Ribo-Zero H/M/R Magnetic Kit (Epicentre, Madison, WI, USA) according to the manufacturer's instructions. A strand-specific library for transcriptome sequencing was prepared using the ScripSeqv2 Kit (Epicentre) following the manufacturer's instructions. The library concentration was determined using an Agilent 2100 Bioanalyzer system with a High Sensitivity DNA Kit (Agilent, Santa Clara, CA, USA) according to the manufacturer's instructions and relying on the concentration of fragments between 200 and 600 nt in size. 19 pmol of library were clustered on one lane of an Illumina paired-end flow cell and 2x100 nt were sequenced according to the manufacturer's instructions using the v3 Cluster Generation and SBS Sequencing Kits (Illumina, San Diego, CA, USA) on a HiSeq2000 system. Adapters were clipped from the sequencing reads using cutadapt (v 1.5)(Martin 2011). Reads were mapped to the human genome version hg19 using TopHat2 (v2.0.13)(Kim et al. 2013). Read counts for Gencode-annotated transcripts were determined using htseqcount (v 0.6.1p1)(Anders et al. 2015) with mode intersection-strict. Counts were normalized and transformed using R and the variance stabilizing transformation of the DESeq2 package (Love et al. 2014) from Bioconductor.

Raw sequencing data with appropriate experimental information is available in the NCBI Gene Expression Omnibus (GEO) repository under the accession number GSE114837 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE114837)

*Statistics*

Statistical significance of parametric distributed values was calculated with Student's t-test. Otherwise, the nonparametric Mann-Whitney U test and Wilcoxon-test were applied for comparison. Boxes in figures indicate the 25 and 75% percentile, whiskers the non-outlier range. Both sensitivity (proportion of positives that are correctly identified as such) and specificity (proportion of negatives that are correctly identified as such) as statistical measures of a binary classification test were calculated based on a cut-off (mean plus one standard deviation) of each T cell subtype. All p-values $<0.05$ were considered to be significant. For multiple testing, the significance level was adjusted according to

Bonferroni. All statistical calculations were performed with Statistica for Windows version 10 (StatSoft Inc. Europe).

## Results

### *GPR15 expression in T cells is uniquely, not accompanied by another protein*

To better describe the physiological role of this cell type we checked first whether expression of other proteins concomitantly follow the GPR15 expression in T cells. As a proxy approach for that, flow cytometric sorted CD3+GPR15+ T cells from peripheral blood were compared with CD3+GPR15- T cells by RNA sequencing (Table S1, Figure 2). The *GPR15* expression was not concomitantly accompanied by expression of another gene neither in the similar range of transcripts (TPM, transcript per million) nor by similar fold change of expression. Additionally, intraindividual differences of randomly selected differentially expressed genes (n, 38) between GPR15- and GPR15+ T cells did not correlate with *GPR15* expression (Table S2). It can therefore be assumed, that the expression of GPR15 at protein level is uniquely, not specifically accompanied by another protein.

Additionally, comparing particular differences of transcript expression by RNAseq with protein expression we could confirm the above assumption. Analyzing surface receptors which were used for identification of specific T cell subtypes, GPR15+ differed from GPR15- T cells by the proportion of receptor-expressing cells (Figure 3, Table S3). GPR15+ T cells contained 1.3-fold ($p<0.001$) more CD4+ Th and 1.9-fold ($p<0.001$) less CD8+ Tc cells. According to chemokine receptors, except for CXCR3, GPR15+ T cells possessed increased proportion of CXCR5 (2.5-fold), CCR4 (3.8-fold), CCR6 (2.4-fold) and CCR10 (3.4-fold) expressing cells ($p<0.001$, each). Consequently, the correlation of transcript expression by RNAseq with proportions of appropriate protein-expressing T cells and not with protein density per each cell confirmed the absence of GPR15 co-expressing proteins.

***Th17 is the most abundant GPR15+ T cell subtype in peripheral blood***

To deeper characterize GPR15+ T cells we analyzed the proportion of GPR15+ cells among different T cell subtypes, including helper (Th), cytotoxic (Tc), regulatory T cells (Treg), follicular Th (Tfh) and mucosa-associated invariant T cells (MAIT), with respect to the smoking behavior (NS, non-smoker; S, chronic active smoker). Irrespective on validated Th lineages, all T cell subtypes which were characterized by the combined expression of CXCR3, CXCR5, CCR4, CCR6 and CCR10 were depicted.

As indicated (Figure 4), the main proportion of GPR15+ T cells (CD3+) in peripheral blood constituted Th (51.6% [49.9% – 53.3%]), followed by less Tfh (25.0% [23.8% – 26.3%]) and Tc (19.7% [17.7% – 21.7%]). Most of the GPR15+ Th cells were central memory Th cells (86.5% [85.0% – 88.0%]). According to different T cell subtypes, there is no single cell type prominently occurred in GPR15+ T cells. With decreasing proportion of subtypes, the CXCR3+CCR4-6-10- Tc (13.0% [11.3% – 14.7%]) followed by Th17 (10.0% [9.3% – 10.8%]) were the first two most frequent GPR15+ T cell subtypes. However, irrespective of differentiation into Th and Tfh subtypes, the Th17 was the most abundant GPR15+ T cell type (17.3% [16.2% - 18.4%]). This cell type was 6.4-fold more frequent compared to GPR15- Th17 cells (2.7% [2.4% - 3.1%], $p<0.001$).

Smoking behavior exerted only minor effect on frequency of T cell subtypes among GPR15+ T cells. In smokers, the proportion of nTreg (9.2% [7.9% – 10.6%], $p<0.001$) and Th2 (6.3% [5.3% -7.2%], $p<0.001$) among GPR15+ T cells was about 1.5-fold higher compared to non-smoker (5.1% [4.4% – 5.8%], 4.2% [3.3% – 5.1%], respectively). In contrast, T cell subtypes of GPR15- lymphocytes, with naive T cells as the most prominent cell type, were not influenced by smoking (Figure S1).

***Smoking induces an excess in each of the GPR15-expressing T cell subtypes in blood***

Independent on smoking behavior, GPR15 was nearly absent in $CD45RA_{high}$ naive T cells and MAIT cells but occurred in all other analyzed T cell subtypes (Figure 5). The abundance of GPR15+ cells differently distinguished in T cell subtypes compared to the whole CD3+ T cell population (NS, 5.1% [confidence interval, 3.9% -6.3%]; S, 13.8% [11.2% -16.3%], p<0.001). The highest GPR15+ cell proportion consisted of Th17 Tfh (NS, 21.2% [16.9% -25.5%]; S, 53.8% [47.4% -60.1%], p<0.001), independent on smoking. Chronic active smoking evoked higher proportion of GPR15+ cells in nearly all GPR15-expressing T cell subtypes compared to non-smoker.

In contrast to the strong smoking-dependent increase in proportion of GPR15+ cells for most of the analyzed differential T cell subtypes, the cell count of T cell subtypes was similar between non-smoker and smoker, except for the slightly higher proportion of CD3+ T cells (68.5% [65.2% – 71.7%]) and the 2.1-fold lower proportion of MAIT cells (4.2% [2.3% – 6.1%]) in smokers compared to non-smokers (63.2% [59.1% – 67.2%, p=0.04], 8.8 [5.2% – 12.3%, p=0.013], respectively)(Figure S2).

***Proportion of GPR15+ T cells in blood is the best specific and sensitive cellular biomarker of smoking***

As depicted in figure 5, the analysis of proportion of GPR15+ cells among T cell subtypes in peripheral blood can be used as a biomarker to distinguish chronic active smoker from non-smoker. By using a cut-off of mean ± one standard deviation, the most specific and sensitive differentiation into non-smoker and chronic smoker was given for Th17 (sensitivity, 1; specificity, 0.91). In contrast, smoking-influenced proportion of MAIT in blood was much less specific to stratify for smoking (sensitivity, 1; specificity, 0.23).

**Discussion**

For the first time, we recently have reported T cells in peripheral blood as the main GPR15+ leucocyte evoking excessively by chronic smoking (Bauer et al. 2015). To get more insight into the physiology as well as pathological importance of GPR15+ T cells we first performed transcriptome analyses by RNAseq and semiquantitative PCR as proxy approaches to identify proteins concomitantly expressed with GPR15. Starting with RNAseq, this approach was done exclusively with separated cell subtypes from one healthy volunteer. Since the focus of this work was not the subject- or exposure-triggered interindividual differences in gene expression, the RNAseq analysis of separated T cells on one subject was sufficient to show co-expressed genes/proteins. Based on the whole transcriptome, the GPR15 surface protein expression seemed to be clearly unique, not accompanied by co-expression of another intracellular or surface protein. This result was substantiated in a greater cohort by (i) a smoking-independent absent correlation between intraindividual *GPR15* expression and randomly selected differentially expressed genes and by (ii) the findings from analyses of surface receptors that, unlike *GPR15,* differences in gene expression between GPR15- and GPR15+ T cells exclusively relied on different proportions of gene-expressing cells but not on a changed cellular abundance of transcripts in each cell.

In blood, we found the distribution and proportion of GPR15+ cells were strikingly influenced by the differentiation state of T cells. The vast majority of about 80% of GPR15+ T cells consisted of diverse subtypes of central memory T cells ($T_{CM}$). A less frequent occurrence of GPR15 was found in cells with effector-like features, with an absence in effector-like MAIT. In contrast to the replicated absence of GPR15+ Treg in the human colon mucosa (Nguyen et al. 2015, Fischer et al. 2015) with the concluding statement about a probable impossibility of GPR15 expression in Treg cells (Nguyen et al. 2015), we and other clearly have shown that proportions of both natural (nTreg (Ocon et al. 2017, Fischer et al. 2015) and active nTreg) and further Tregs (Tr1, Th3) in blood were able to express GPR15. Irrespective of the presence of GPR15+ cells among nearly all analyzed T cell subtypes (Th1, Th2, Th9, Th17, Th22 et al.), the most abundant GPR15+ T

cells were Th17. This overrepresentation should be the reason for an increased IL-17 secretion of GPR15+ CD4+ T cells in healthy PBMCs in comparison to GPR15- T cells (Adamczyk et al. 2017).

To date, the physiological mechanism to release GPR15+ lymphoid cells into blood remains unknown. First changes in proportion of GPR15+ cells among T cell subtypes of blood were reported exclusively for Treg in patients with ulcerative colitis (Fischer et al. 2015). However, due to the marginal (difference between number of GPR15+ Treg in non-UC and UC patients, <10%) and interindividual changes the proportion of GPR15+ Treg cannot serve as a clinical biomarker for UC. Chronic tobacco smoking is to date the only known specific inducer for an excess of GPR15+ cells in blood (Bauer et al. 2015, Bauer et al. 2015). We now have shown that this excess was not restricted to any GPR15+ expressing T cell subtype in blood. The extent of GPR15+ cells individually reached values of up to 80% in single T cell subtypes, especially in Th17. A degree of proportion similar found after HIV-1 infection of a CD4+ T-cell clone (PM1, up to about 60% of infected cells) *in vitro* (Kiene et al. 2014) or in seemingly natural occurring CD25+CD127$^{high}$FoxP3- Th in mucosa of the colon (Nguyen et al. 2015).

Because of the strong association of GPR15 expression with epigenetic DNA methylation at CpG site cg19859270 (annotated to *GPR15*), it can be assumed from epigenome-wide association studies (EWAS) that the excess of GPR15+ cells in blood increases linearly with increasing smoking dosage (pack years, (Ambatipudi et al. 2016)). In our study we could not prove this expectation because of missing data about smoking dosage. A sustained T cell stimulation critically dependents on a continuous supply of antigen-carrying DCs (Lanzavecchia and Sallusto 2000). Therefore, it was recently postulated that the tobacco smoking-induced disturbance of homeostasis, primarily in the lung, might be the origin to imprint T cells by activated antigen-presenting dendritic cells (DC). However, a smoking-independent disturbed homeostasis by different lung pathologies did not evoke an excess of GPR15+ T cells in blood in never-smoking patients (Bauer et al. 2017). Similarly, a mucosal inflammation did not affect the content of GPR15+ effector memory Th cells in inflamed colon mucosa (Nguyen et al. 2015). Whether GPR15 induction is

influenced by water-soluble compounds of cigarette smoke remains speculative, but could not have been confirmed by a simple exposure of PBMCs to an aqueous cigarette smoke extract in an artificial *in-vitro* approach (Bauer et al. 2015).

Apart from the cellular response on smoking by augmentation of GPR15+ T cells, it has long been known that smoking impairs myeloid (Friedman et al. 1973, Petitti and Kipp 1986) and lymphoid cell (Moszczynski et al. 2001, Ammitzboll et al. 2017) count in whole blood. With respect to T cell subtypes we could confirm the recently described smoking-induced reduction of mucosal associated invariant T cell (MAIT) content in blood (Ammitzboll et al. 2017). Interestingly, that one T cell subtype which not expressed GPR15. However, because of several MAIT-depleting adverse physical conditions in peripheral blood (Hinks et al. 2015, Serriari et al. 2014, Bottcher et al. 2018) the abundance of MAIT was less specific in comparison to the proportion of GPR15+ T cells to discriminate subjects for smoking behavior. Since the content of MAIT was reduced both in blood and liver tissue in non-infectious liver pathologies, their reduction was consequently proposed to be rather a sign of exhaustion induced by chronic activation, a mechanism seemingly similar evoked by chronic smoking. In contrast, the smoking-induced excess of GPR15+ T cells in blood is probably more exposure-specific, therefore applicable as a convincing biomarker for chronic smoking reaching maximal sensitivity and specificity of 1 each (Bauer et al. 2017). In this study we obtained only a suboptimal specificity of 0.91 since we could not discriminate non-smoker from never smoker by missing questionnaire for that.

Because of the migratory activity of seemingly each GPR15-expressing T cells in blood, including Treg, Tmemory, Teffector, towards GPR15L (Ocon et al. 2017), it can be presumed that, acting as a homing receptor, a similar diverse pattern of GPR15+ T cell subtypes in peripheral blood occurs at the site of homing, such as epidermis or colon mucosa, with an expected slightly overrepresentation of Th17 cells. The prominent presence of Th17 at sites of tissue inflammation such as psoriasis and inflammatory bowel

disease (Korn et al. 2009) as well as high proportion of IL-17-secreting GPR15+ memory Th cells in UC mucosa would confirm this assumption (Nguyen et al. 2015). However, similar to the differentiation state of T cells the microenvironments should decisively influence GPR15 expression and cell type specific homing. (i) The higher, compared to normal, extent of GPR15+ cells in blood by chronic smoking, (ii) the repressed GPR15 expression of Treg in human colon mucosa but not in blood, or (iii) the induced GPR15 expression under artificial polarizing conditions *in vitro (Nguyen et al. 2015)* underline the role of tissue-specific microenvironment to influence the abundance of GPR15+ cells. Irrespective of GPR15, the final role of Th17 is decisively triggered by the local microenvironment. It may generate either non-pathogenic or pathogenic Th17 cells. Interestingly, under non-pathogenic conditions the *GPR15* was about 1.9-fold overexpressed in Th17 cells (Lee et al. 2012), what might account for a doubling of abundance of GPR15+ Th17 cells under non-pathogenic conditions.

Additionally, since GPR15-deficient mice were prone to develop severe large intestine inflammation (Kim et al. 2013) a similar preventive role of GPR15-expressing cells was herewith documented. Altogether, GPR15 might be considered more as a sign of protection from harm by inflammation than a harm by itself. In healthy chronic smoking subjects, therefore, the pronounced GPR15 expression in blood might, irrespective to be an indicator of adverse effects, be important to prevent harm from smoking.



**Conclusion**

In this report we highlighted the strong impact of chronic smoking on the imprinting of T cells for GPR15 occurring high-frequently in vascular circulation. Whether smoking-induced enrichment of GPR15+ lymphocytes in blood or appropriate tissue has a health-preventing effect of smoking for patients suffering from ulcerative colitis (Bridger et al. 2002) or, in opposite, has a health-threatening effect of smoking for patients suffering from incident psoriasis (Naldi 2016) could not be resolved in this study. Indeed, severe large intestine inflammation was rescued by the transfer of GPR15-sufficient Treg indicating that GPR15 plays a role in mucosal immune tolerance, at least in mice (Kim et al. 2013).

Therefore, whether the GPR15L-GPR15 interaction may be an option for systemic therapeutic intervention needs to be proven in human. In mice, first evidence was given by systemic application of a GPR15L in form of the fifth region of epidermal growth factor–like domain of TM (TME5) that alleviated an acute graft-versus-host disease by its proposed anti-inflammatory impact (Pan et al. 2017).

However, because of the naturally occurring GPR15+ lymphocytes in blood of healthy subjects and the packyears-dependent increased presence of GPR15+ lymphocytes in blood of healthy smokers we suggest that GPR15+ lymphocytes in blood of smokers constitute rather a sign of adaptation to prevent harm particularly from tobacco smoking. Therefore, it should be proven whether therapeutic intervention of the GPR15-GPR15L interaction will be applicable in smokers, too.

**Acknowledgements**

We thank Elke Gille and Gulja Willnauer from the Institute of transfusion medicine at the University of Leipzig for their support in provision of data from voluntary blood donors. We thank Kathrin Jäger and Andreas Lösche for performing the flow-cytometric cell sorting at the laboratory of cytometry of the Core Facility at the University of Leipzig.

**Disclosure assessment**

No potential conflict of interest was reported by the authors.

## References


Ocon B, Pan J, Dinh TT, Chen W, Ballet R, Bscheider M, Habtezion A, Tu H, Zabel BA and Butcher EC. (2017). A Mucosal and Cutaneous Chemokine Ligand for the Lymphocyte Chemoattractant Receptor GPR15. *Front Immunol* 8:1111.

Guo P, Luo Y, Mai G, Zhang M, Wang G, Zhao M, Gao L, Li F and Zhou F. (2014). Gene expression profile based classification models of psoriasis. *Genomics* 103:48-55.

Deng HK, Unutmaz D, KewalRamani VN and Littman DR. (1997). Expression cloning of new receptors used by simian and human immunodeficiency viruses. *Nature* 388:296-300.

Kim SV, Xiang WV, Kwak C, Yang Y, Lin XW, Ota M, Sarpel U, Rifkin DB, Xu R and Littman DR. (2013). GPR15-mediated homing controls immune homeostasis in the large intestine mucosa. *Science* 340:1456-9.

Lahl K, Sweere J, Pan J and Butcher E. (2014). Orphan chemoattractant receptor GPR15 mediates dendritic epidermal T-cell recruitment to the skin. *Eur J Immunol* 44:2577-81.

Nguyen LP, Pan J, Dinh TT, Hadeiba H, O'Hara E, 3rd, Ebtikar A, Hertweck A, Gokmen MR, Lord GM, Jenner RG, Butcher EC and Habtezion A. (2015). Role and species-specific expression of colon T cell homing receptor GPR15 in colitis. *Nat Immunol* 16:207-13.

Fischer A, Zundler S, Atreya R, Rath T, Voskens C, Hirschmann S, Lopez-Posadas R, Watson A, Becker C, Schuler G, Neufert C, Atreya I and Neurath MF. (2015). Differential effects of alpha4beta7 and GPR15 on homing of effector and regulatory T cells from patients with UC to the inflamed gut in vivo. *Gut*.

Coda AB, Icen M, Smith JR and Sinha AA. (2012). Global transcriptional analysis of psoriatic skin and blood confirms known disease-associated pathways and highlights novel genomic "hot spots" for differentially expressed genes. *Genomics* 100:18-26.

Bauer M, Linsel G, Fink B, Offenberg K, Hahn AM, Sack U, Knaack H, Eszlinger M and Herberth G. (2015). A varying T cell subtype explains apparent tobacco smoking induced single CpG hypomethylation in whole blood. *Clin Epigenetics* 7:81.

Bauer M, Fink B, Seyfarth HJ, Wirtz H and Frille A. (2017). Tobacco-smoking induced GPR15-expressing T cells in blood do not indicate pulmonary damage. *BMC Pulm Med* 17:159.

Koks G, Uudelepp ML, Limbach M, Peterson P, Reimann E and Koks S. (2015). Smoking-Induced Expression of the GPR15 Gene Indicates Its Potential Role in Chronic Inflammatory Pathologies. *Am J Pathol* 185:2898-906.

Ambatipudi S, Cuenin C, Hernandez-Vargas H, Ghantous A, Le Calvez-Kelm F, Kaaks R, Barrdahl M, Boeing H, Aleksandrova K, Trichopoulou A, Lagiou P, Naska A, Palli D, Krogh V, Polidoro S, Tumino R, Panico S, Bueno-de-Mesquita B, Peeters PH, Quiros JR, Navarro C, Ardanaz E, Dorronsoro M, Key T, Vineis P, Murphy N, Riboli E, Romieu I and Herceg Z. (2016). Tobacco smoking-associated genome-wide DNA methylation changes in the EPIC study. *Epigenomics* 8:599-618.

Livak KJ and Schmittgen TD. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25:402-8.

Hackermuller J, Reiche K, Otto C, Hosler N, Blumert C, Brocke-Heidrich K, Bohlig L, Nitsche A, Kasack K, Ahnert P, Krupp W, Engeland K, Stadler PF and Horn F. (2014). Cell cycle, oncogenic and tumor suppressor pathways regulate numerous long and macro non-protein-coding RNAs. *Genome Biol* 15:R48.

Martin M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnetjournal* 17.

Kim D, Pertea G, Trapnell C, Pimentel H, Kelley R and Salzberg SL. (2013). TopHat2: accurate alignment of transcriptomes in the presence of insertions, deletions and gene fusions. *Genome Biol* 14:R36.
Anders S, Pyl PT and Huber W. (2015). HTSeq--a Python framework to work with high-throughput sequencing data. *Bioinformatics* 31:166-9.
Love MI, Huber W and Anders S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol* 15:550.
Adamczyk A, Gageik D, Frede A, Pastille E, Hansen W, Rueffer A, Buer J, Buning J, Langhorst J and Westendorf AM. (2017). Differential expression of GPR15 on T cells during ulcerative colitis. *JCI Insight* 2.
Bauer M, Fink B, Thurmann L, Eszlinger M, Herberth G and Lehmann I. (2015). Tobacco smoking differently influences cell types of the innate and adaptive immune system-indications from CpG site methylation. *Clin Epigenetics* 7:83.
Kiene M, Rethi B, Jansson M, Dillon S, Lee E, Lantto R, Wilson C, Pohlmann S and Chiodi F. (2014). Toll-like receptor 3 signalling up-regulates expression of the HIV co-receptor G-protein coupled receptor 15 on human CD4+ T cells. *PLoS One* 9:e88195.
Lanzavecchia A and Sallusto F. (2000). Dynamics of T lymphocyte responses: intermediates, effectors, and memory cells. *Science* 290:92-7.
Friedman GD, Siegelaub AB, Seltzer CC, Feldman R and Collen MF. (1973). Smoking habits and the leukocyte count. *Arch Environ Health* 26:137-43.
Petitti DB and Kipp H. (1986). The leukocyte count: associations with intensity of smoking and persistence of effect after quitting. *Am J Epidemiol* 123:89-95.
Moszczynski P, Zabinski Z, Moszczynski P, Jr., Rutowski J, Slowinski S and Tabarowski Z. (2001). Immunological findings in cigarette smokers. *Toxicol Lett* 118:121-7.
Ammitzboll C, Bornsen L, Romme Christensen J, Ratzer R, Romme Nielsen B, Sondergaard HB, von Essen MR and Sellebjerg F. (2017). Smoking reduces circulating CD26hiCD161hi MAIT cells in healthy individuals and patients with multiple sclerosis. *J Leukoc Biol* 101:1211-20.
Hinks TS, Zhou X, Staples KJ, Dimitrov BD, Manta A, Petrossian T, Lum PY, Smith CG, Ward JA, Howarth PH, Walls AF, Gadola SD and Djukanovic R. (2015). Innate and adaptive T cells in asthmatic patients: Relationship to severity and disease mechanisms. *J Allergy Clin Immunol*:doi: 10.1016/j.jaci.2015.01.014.
Serriari NE, Eoche M, Lamotte L, Lion J, Fumery M, Marcelo P, Chatelain D, Barre A, Nguyen-Khac E, Lantz O, Dupas JL and Treiner E. (2014). Innate mucosal-associated invariant T (MAIT) cells are activated in inflammatory bowel diseases. *Clin Exp Immunol* 176:266-74.
Bottcher K, Rombouts K, Saffioti F, Roccarina D, Rosselli M, Hall A, Luong T, Tsochatzis EA, Thorburn D and Pinzani M. (2018). MAIT cells are chronically activated in patients with autoimmune liver disease and promote pro-fibrogenic hepatic stellate cell activation. *Hepatology*.
Korn T, Bettelli E, Oukka M and Kuchroo VK. (2009). IL-17 and Th17 Cells. *Annu Rev Immunol* 27:485-517.
Lee Y, Awasthi A, Yosef N, Quintana FJ, Xiao S, Peters A, Wu C, Kleinewietfeld M, Kunder S, Hafler DA, Sobel RA, Regev A and Kuchroo VK. (2012). Induction and molecular signature of pathogenic TH17 cells. *Nat Immunol* 13:991-9.
Bridger S, Lee JC, Bjarnason I, Jones JE and Macpherson AJ. (2002). In siblings with similar genetic susceptibility for inflammatory bowel disease, smokers tend to develop Crohn's disease and non-smokers develop ulcerative colitis. *Gut* 51:21-5.
Naldi L. (2016). Psoriasis and smoking: links and risks. *Psoriasis (Auckl)* 6:65-71.
Pan B, Wang X, Kojima S, Nishioka C, Yokoyama A, Honda G, Xu K and Ikezoe T. (2017). The Fifth Epidermal Growth Factor-like Region of Thrombomodulin Alleviates Murine Graft-versus-Host

Disease in a G-Protein Coupled Receptor 15 Dependent Manner. *Biol Blood Marrow Transplant* 23:746-56.

## Figure Legends

Figure 1. Flow diagram for participants of the study.

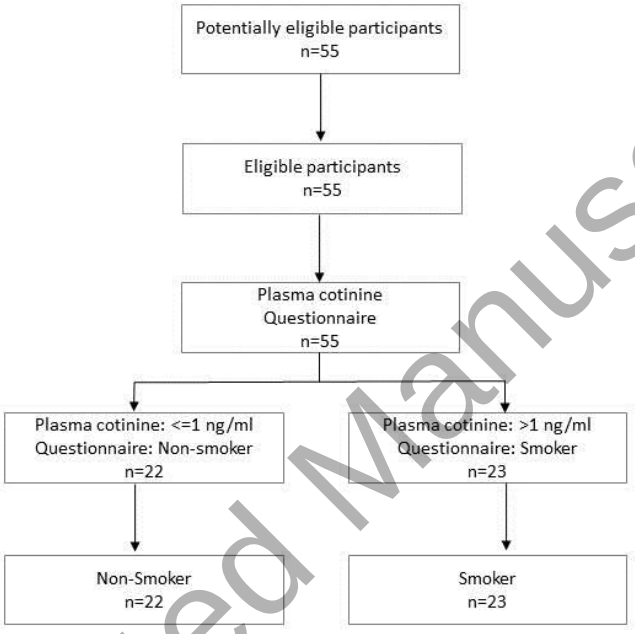


Figure 1

Figure 2. Differences of gene expression between GPR15+ and GPR15- T cells (CD3+) from peripheral blood of healthy adult volunteer, analyzed by RNAseq. In T cells, *GPR15* is uniquely expressed, not accompanied by another gene neither in the similar range of transcripts (TPM) nor by similar fold change of expression. TPM, unit of transcript expression (Transcripts Per Million); FC, x-fold change.

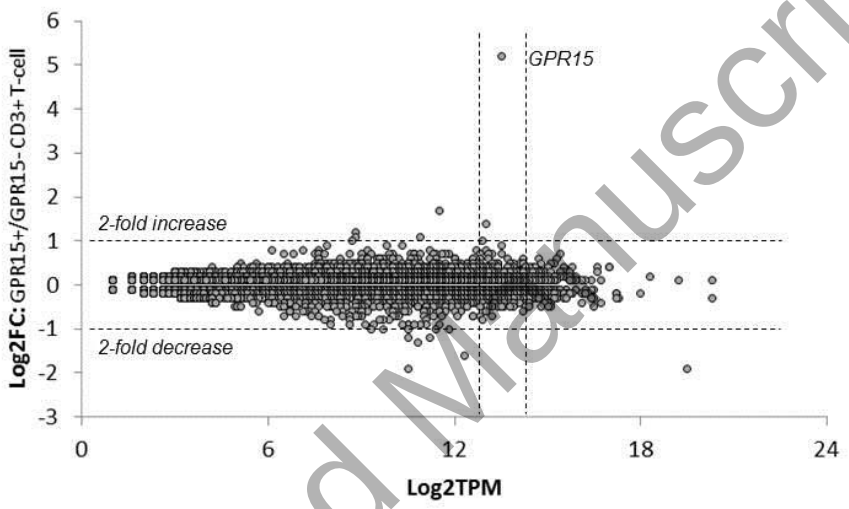


Figure 2

Figure 3. Proportion of surface receptor-expressing cells in GPR15- and GPR15+ T cells considering smoking behavior. GPR15+ differs from GPR15- T cells by the proportion of receptor-expressing cells and not by protein density per each cell confirming the absence of GPR15 co-expressing proteins suggested by transcriptome analyses. Asterisks indicate difference between GPR15- and GPR15+ T cells ($p<0.001$).

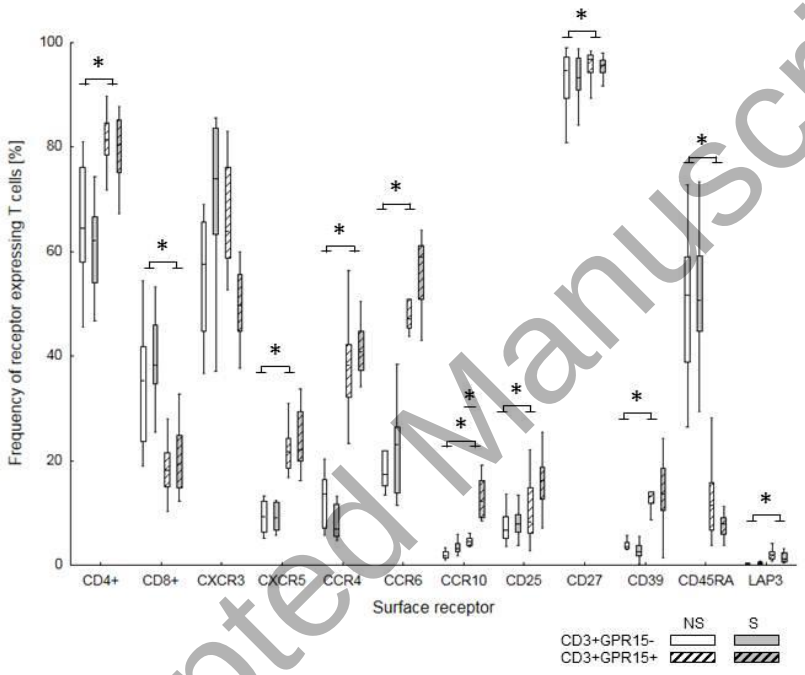


Figure 3

Figure 4. Different proportion of T cell subtypes among GPR15+ T cells in adult non-smokers and smokers. Most dominant GPR15+ T cells are central memory helper T cells. From T cell subtypes, the CXCR3+ cytotoxic T cell is the most abundant T cell subtype, followed by Th17. The numbers 3, 4, 5, 6 and 10 in T cell subtypes indicate CXCR3-, CCR4-, CXCR5-, CCR6- and CCR10-surface receptors. +,- indicates presence or absence of surface receptor. Asterisks indicate significant difference between non-smoker and smoker for $p<0.001$ (Student's t-test, expected p-value for multiple testing of 0.00116).

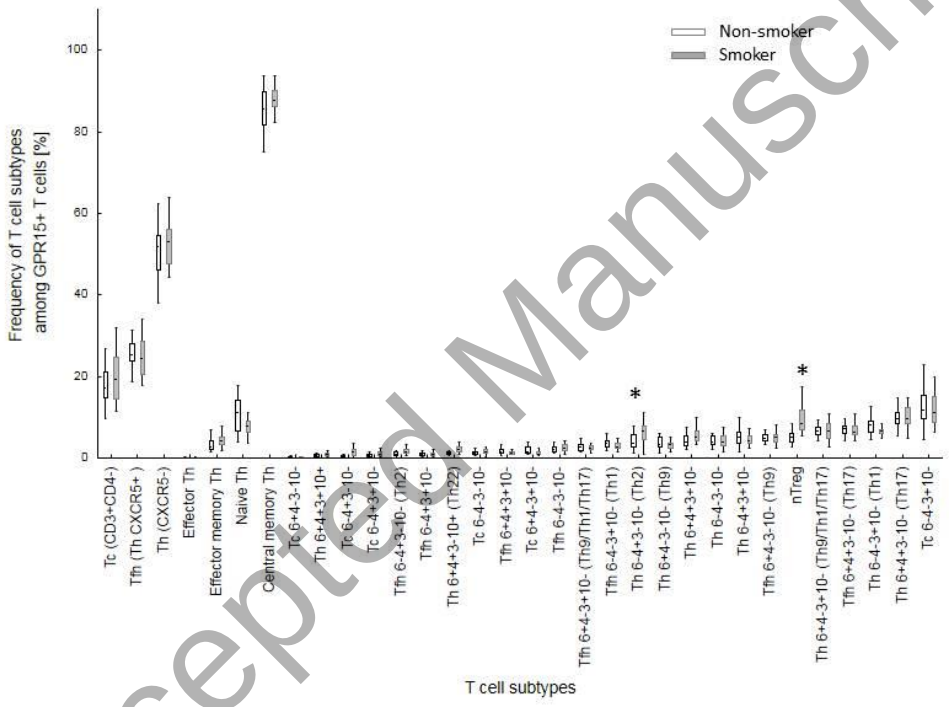


Figure 4

Figure 5. Different proportion of GPR15+ cells among different T cell subtypes in adult non-smokers and smokers. GPR15 expression is nearly absent in naïve Th and CD8+ MAIT cells. Activation of GPR15 expression in smokers is not restricted to a single T cell subtype. The highest proportion of GPR15+ cells is found in Th17 cells. Asterisks indicate significant difference between non-smoker and smoker for $p<0.0001$ (Student's t-test, expected p-value for multiple testing of 0.00116).

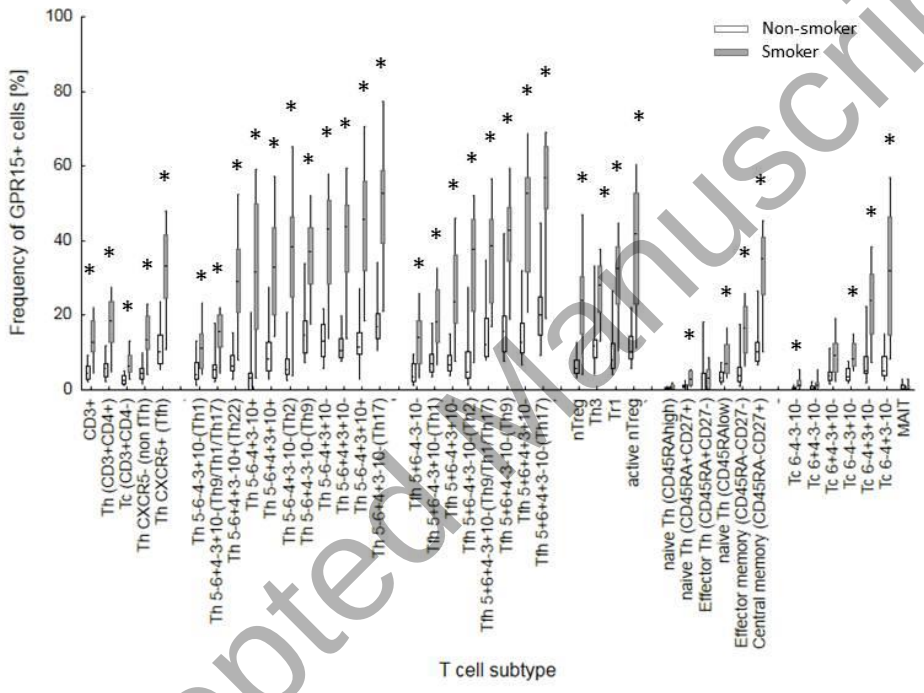


Figure 5