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The expression profile of the ubiquitin-like modifier FAT10 in immune cells suggests cell type-specific functions

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Abstract
The TNF and IFN-γ-inducible ubiquitin-like modifier HLA-F adjacent transcript 10 (FAT10) is most prominently expressed in immunological tissues but information regarding basal expression and inducibility of FAT10 in the different types of immune cells is still lacking. Hence, we investigated *FAT10* mRNA expression in the major human and murine immune cell subsets, and FAT10 protein expression in human leukocytes. We isolated the different human leukocytes from peripheral blood and the murine immune cell subsets from spleen. The purified leukocytes were left untreated or stimulated with TNF and INF-γ or LPS to induce *FAT10* followed by quantitative real-time PCR or western blot analysis. Basal expression of *FAT10* mRNA and protein was generally low but strongly up-regulated by IFN-γ and TNF in all immune cell subsets. LPS treatment induced *FAT10* expression marginally in human $CD8^+$ T cells and murine granulocytes, but it increased *Fat10* expression significantly in murine regulatory T cells. Yet, in human $CD8^+$ T cells, natural killer cells, natural killer T cells, and dendritic cells, the *FAT10* mRNA was expressed without induction. Similarly, murine macrophages, monocytes, and regulatory T cells expressed *Fat10* in the absence of stimulation. In summary, our findings suggest particular functions of FAT10 in these cell types. Furthermore, we observed not only a cell type-specific but also a species-specific basal *FAT10* expression profile. Our data will serve as a guideline for future investigations to further elucidate FAT10's role in the immune system.

Keywords FAT10 · Ubiquitin-like modifier · Interferon-γ · Tumour necrosis factor · Expression level

Introduction

FAT10 is a ubiquitin-like protein that was discovered by chromosomal sequencing in an effort to identify additional genes within the human MHC locus. Therefore, it was designated according to its proximity to the HLA-F locus 'HLA-F adjacent transcript 10' (FAT10) (Fan et al. 1995, 1996). FAT10 consists of two ubiquitin-like domains, which are joined by a short linker. The N- and C-terminal ubiquitin-like domains of FAT10 share 29 and 36% sequence identity with ubiquitin, respectively. In contrast to ubiquitin, FAT10 is synthesised as a mature protein with an already accessible diglycine motif at its C-terminus (Bates et al. 1997). The diglycine motif is essential for the isopeptide linkage of FAT10 to many different substrates, a process termed FAT10ylation (Raasi et al. 2001; Aichem et al. 2012). Similar to ubiquitin, an enzymatic cascade comprising an E1, E2, and possibly an E3 enzyme mediates covalent attachment of FAT10. Here, UBA6 (ubiquitin-activating enzyme 6) (Jin et al. 2007; Pelzer et al. 2007) and USE1 (UBA6-specific E2 enzyme 1) (Aichem et al. 2010) serve as E1 activating and E2 conjugating enzymes, respectively. Both enzymes are bispecific and can activate FAT10 and ubiquitin (Chiu et al. 2007; Aichem et al. 2010). Similar to ubiquitin, FAT10 leads to degradation of covalently bound proteins by the proteasome (Raasi et al. 2001; Hipp et al. 2005; Schmidtke et al. 2014). Yet, FAT10 is probably degraded by the proteasome along with its substrates instead of being cleaved by specific de-conjugating enzymes (Hipp et al. 2005; Aichem et al. 2014).

Thus far, conjugation and proteasomal targeting of FAT10 are reasonably well understood, while the biological functions of FAT10 have remained less well defined. FAT10 is involved in multiple cellular processes like apoptosis (Liu et al. 1999; Raasi et al. 2001; Ross et al. 2006), mitosis (Liu

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et al. 1999; Ren et al. 2006, 2011; Merbl et al. 2013), and NF-κB activation (Gong et al. 2010). Moreover, FAT10 plays a role in defence against intracellular bacteria (Spinnenhirn et al. 2014) and antigen processing (Ebstein et al. 2012; Schliehe et al. 2012), influences negative selection, and alters antigen presentation in the thymus (Buerger et al. 2015). High basal expression of *Fat10* mRNA is predominantly found in organs of the immune system like the thymus, foetal liver, lymph nodes, and spleen (Lee et al. 2003; Canaan et al. 2006; Lukasiak et al. 2008). So far, endogenous *FAT10* expression at the cellular level has been assigned to mature dendritic cells (DC) and B cells (Bates et al. 1997), medullary thymic epithelial cells (Buerger et al. 2015), and to long-term cultures of human regulatory T cells (Treg) (Ocklenburg et al. 2006). Notably, expression of FAT10 can be synergistically induced by the pro-inflammatory cytokines IFN-γ and TNF as well as IL-6 and TNF in a variety of cells (Liu et al. 1999; Raasi et al. 1999; Choi et al. 2014) and *FAT10* expression in cancer tissues correlated with other cytokine-induced genes (Lukasiak et al. 2008). *FAT10* expression can also be induced in in vitro-generated DC and mature B cells by other stimuli, like lipopolysaccharides (LPS), CD40L, polyI:C, and a cocktail of several pro-inflammatory cytokines (Bates et al. 1997; Lukasiak et al. 2008; Ebstein et al. 2009). In addition, *FAT10* expression is suppressed by p53 (Zhang et al. 2006), and high *FAT10* expression was detected in several tumours like in hepatocellular carcinoma, colon carcinoma, glioma, ovarian and uterus carcinomas, suggesting a role of FAT10 in neoplastic processes (Lee et al. 2003).

Tissue-specific expression of *FAT10* has been well defined (Lee et al. 2003; Canaan et al. 2006, 2014; Lukasiak et al. 2008) showing a clear preference towards tissues of the immune system or under inflammatory conditions. However, there is scarce information about *FAT10* expression in the different immune cell types. Hence, we have investigated the expression levels of *FAT10* mRNA in the main human and murine leukocyte populations of the adaptive and innate immune system in their naïve state, upon stimulation with pro-inflammatory cytokines, and endotoxin treatment. Moreover, FAT10 protein expression was examined in cytokine-stimulated and unstimulated human peripheral blood mononuclear cells (PBMCs). This data set will be instructive for the direction of future research on FAT10 in order to disclose its proper function in the immune system and tumour biology.

Materials and methods

Mice

C57BL/6 mice (H-2^b) were originally purchased from Charles River Laboratories (Germany). C57BL/6 Foxp3-GFP reporter mice were kindly provided by H.C. Probst (Lahl et al. 2007) and FAT10-deficient mice (Canaan et al. 2006) by A. Canaan and S. M. Weissman (Yale University School of Medicine, New Haven, CT). Mice were kept in a specific pathogen-free facility. Sex- and age-matched mice were used at 8–10 weeks of age. All mouse experiments were approved by the review board of Regierungspräsidium Freiburg.

Peripheral blood mononuclear cell and granulocyte isolation

Blood donations for research purposes were approved by the Ethics Committee of Konstanz University, and individual donors gave written consent. PBMCs from healthy donors were enriched by density gradient centrifugation on Ficoll-Paque™ Plus (GE Healthcare, Germany). Remaining red blood cells were lysed in ACK buffer (8.29 g/L NH_4Cl, 1 g/L $KHCO_3$, 0.1 mM EDTA) for 5 min at room temperature. Granulocytes were isolated from the fraction containing polymorphonuclear cells (PMNCs) and erythrocytes after density centrifugation. To remove as many erythrocytes as possible, the cells were lysed twice consecutively in ACK buffer prior to sorting.

Magnetically activated cell sorting

Magnetically activated cell sorting (MACS) was performed according to the manufacturer's instructions (Miltenyi Biotech, Germany). The MACS kits used are listed in Table 1.

Human PBMCs were sorted sequentially for monocytes, B cells, natural killer (NK) and NKT cells, dendritic cells, and pan-T cells. Finally, human T regulatory cells, $CD8^+$, and $CD4^+$ T cells were separated from pan-T cells using the $CD4^+CD25^+$ Regulatory T Cell Isolation Kit. Human and murine T regulatory cells were further purified by fluorescence-activated cell sorting (FACS) (see below).

Flow cytometry

To isolate Foxp3-GFP positive cells from mice, the following procedure was followed: splenocytes from C57BL/6 Foxp3-GFP reporter mice were first sorted using autoMACS® Pro Separator (Miltenyi Biotech, Germany). The enriched $CD4^+$ T cells were stained with APC-conjugated anti-CD4 (GK1.5) (Biolegend, UK) and GFP^+CD4^+ cells were sorted with a BD FACSAria lllu cell sorter (BD Biosciences, Germany). Human T regulatory cells were first sorted from PBMCs by MACS on the autoMACS® Pro Separator (Miltenyi Biotech, Germany). Then, the cells were stained with APC-conjugated anti-CD25 (4E3) (Miltenyi Biotech, Germany), BV785-conjugated anti-CD4 (RPA-T4), BV421-conjugated anti-

Table 1 MACS kits used to isolate immune cell subsets

MACS kit	Species	Cell type
Anti-Ly-6G MicroBead Kit	Mouse	Granulocytes
CD4 MicroBeads	Mouse	$CD4^+$ T cells
CD8 MicroBeads	Mouse	$CD8^+$ T cells
CD11b MicroBeads	Mouse	Macrophages and monocytes
CD11c MicroBeads	Mouse	Dendritic cells
CD19 MicroBeads	Mouse	B cells
$CD4^+$ T Cell Isolation Kit	Mouse	Regulatory T cells
CD15 MicroBeads	Human	Granulocytes
CD14 MicroBeads	Human	Monocytes
CD19 MicroBeads	Human	B cells
CD56 MicroBeads	Human	NK and NKT cells
Blood Dendritic Cell Isolation Kit II	Human	Dendritic cells
Pan T Cell Isolation Kit	Human	Pan-T cells
$CD4^+CD25^+$ Regulatory T Cell Isolation Kit	Human	$CD4^+$, $CD8^+$, and regulatory T cells

CD357 [GITR] (108-17), and BV605-conjugated anti-CD127 [IL-7Rα] (A019D5) antibodies (BioLegend, UK) and were sorted using a BD FACSAria lllu cell sorter. Dead cells were excluded by Fixable Viability Dye eFluor 520 (Thermo Fisher Scientific, Germany) staining. Successful isolation by FACS was tested on a BD FACSAria lllu instrument. Purity of regulatory T cells was judged by measuring the percentage of GFP^+ cells for murine cells and percentage of $Foxp3^+$ cells in case of human cells. We used the Foxp3 Transcription Factor Staining Buffer Kit (Thermo Fisher Scientific, Germany) to stain intracellular Foxp3 according to the manufacturer's instructions, except that we incubated cells overnight with PE-conjugated anti-Foxp3 (236A/E7) antibody (Thermo Fisher Scientific, Germany). Antibodies used to test successful isolation of immune cells by MACS are listed in Table 2 for human cells and in Table 3 for murine cells.

The purity of sorted immune cell subsets was analysed on an Accuri C6 flow cytometer (BD Biosciences, Germany).

Table 2 Antibodies used to check purity of the cells isolated from human PBMCs

Antibody (clone)	Conjugate(s)	Supplier
Anti-CD3 (HIT3a)	FITC	BD Biosciences (Germany)
Anti-CD4 (RPA-T4)	APC, FITC	BD Biosciences (Germany)
Anti-CD8 (SK1)	APC	BD Biosciences (Germany)
Anti-CD14 (TÜK4)	FITC	BD Biosciences (Germany)
Anti-CD15 (HI98)	FITC	BD Biosciences (Germany)
Anti-CD19 (HIB19)	APC	BD Biosciences (Germany)
Anti-CD56 (B159)	APC	BD Biosciences (Germany)
Anti-HLA-DR (G46-6)	FITC	BD Biosciences (Germany)
Anti-CD303 (201A)	APC	BioLegend (UK)

Cell culture and in vitro stimulation

The sorted mouse immune cell subsets and HEK293T cells (ATCC, USA) were cultured in Iscove's Modified Dulbecco's Medium (IMDM) with GlutaMAX™ supplemented with 10% FCS, 100 U/mL penicillin, and 100 mg/mL streptomycin. Human immune cell subsets were cultured in AIM-V™ medium supplemented with 2% heat-inactivated human AB serum (Sigma-Aldrich, USA) and 50 μM β-mercaptoethanol. Culture media and supplements were bought from Thermo Fisher Scientific (Germany) unless otherwise stated. Cells were stimulated with either 1 μg/mL LPS (Sigma-Aldrich, Germany), or with 400 U/mL TNF and 200 U/mL IFN-γ (both from PeproTech, Germany) for 24 h.

Quantitative real-time RT-PCR

RNA was purified using the RNeasy® Plus Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. For synthesis of single-stranded cDNA from total RNA, the Reverse Transcription System (Promega, Germany) was used. Relative gene expression was measured using the LightCycler® Fast Start DNA Master SYBR Green I kit (Roche, Germany). Mouse samples were analysed on the LightCycler® instrument with the LightCycler® Software Version 3.5 (both from Roche, Germany). The TOptical Gradient 96 Real-Time PCR-Thermocycler and the qPCRsoft V3.1 software (both from Analytik Jena, Germany) were utilised for human samples. The primers we used in the RT-PCR reaction are listed in Table 4.

PCR programs in the LightCycler® software were set up as described previously (Buerger et al. 2015). Settings for the initial denaturation and amplification cycles in the qPCRsoft

Table 3 Antibodies used to assess purity of isolated murine leukocyte subsets

Antibody (clone)	Conjugate	Supplier
Anti-CD4 (GK1.5)	APC	Biolegend (UK)
Anti-CD8a (53-6.7)	APC	Thermo Fisher Scientific (Germany)
Anti-CD11b (M1/70)	PE	Thermo Fisher Scientific (Germany)
Anti-GR-1 (RB6-8C5)	FITC	Thermo Fisher Scientific (Germany)
Anti-CD11c (HL3)	APC	BD Biosciences (Germany)
Anti-CD19 (1D3)	PE	BD Biosciences (Germany)

software were identical to the LightCycler® software, except for a 5 °C/s transition rate. However, in order to assess the specificity of PCR products using the qPCRsoft software, the melting curve was recorded from 60 to 95 °C with 1 °C increments at a transition rate of 5 °C/s, and the fluorescence was detected after an equilibration time of 6 s at each step. Relative gene expression was calculated using the Excel-based relative expressions software tool (REST©) according to the Pfaffl method (Pfaffl et al. 2002).

Immunoprecipitation and western blotting

Approximately 8×10^8 PBMCs from two healthy donors were isolated by Ficoll gradient separation, split into two portions, and cultivated for 30 h in RPMI1640, supplemented with 2% human AB serum in presence or absence of 5000 U/mL TNF and 2500 U/mL IFN-γ, as described earlier (Aichem et al. 2010). Cells were harvested and lysed in RIPA buffer, supplemented with protease inhibitors (complete EDTA-free protease inhibitor cocktail, Roche, Germany). Cleared lysates were subjected to immunoprecipitation using the FAT10-reactive monoclonal antibody 4F1 coupled to agarose, as previously described (Aichem et al. 2010). Proteins were separated on NuPAGE 4–12% gradient gels (Thermo Fisher Scientific, Germany) and subjected to western blot analysis using a FAT10-reactive polyclonal antibody (Hipp et al. 2005). β-Actin was used as loading control.

Table 4 Primer pairs for quantitative RT-PCR

mRNA	Forward (5′ to 3′)	Reverse (5′ to 3′)
(MOUSE) Fat10	GGGATTGACAAGGA AACCACTA	TTCACAACCTGCTT CTTAGGG
(MOUSE) Rpl13a	TGAAGGCATCAACA TTTCTGG	GGTAAGCAAACTTT CTGGTAG
(MOUSE) Actb	GACCTCTATGCCAA CACAGT	ACTCATCGTACTCC TGCTTG
(HUMAN) FAT10	CTGTGTGCATGTCC GTTCCGA	GGGTAAGGTGGATG GTCTTCTCT
(HUMAN) RPL13A	CTACAGAAACAAGT TGAAGTACCTG	ATGCCGTCAAACAC CTTGAG
(HUMAN) SHDA	GATTACTCCAAGCC CATCCA	CACAGTCAGCCTCG TTCAAA
(HUMAN) TBP	GTAAACTTGACCTA AAGACCATTGC	CTGTTCTTCACTCT TGGCTCCT

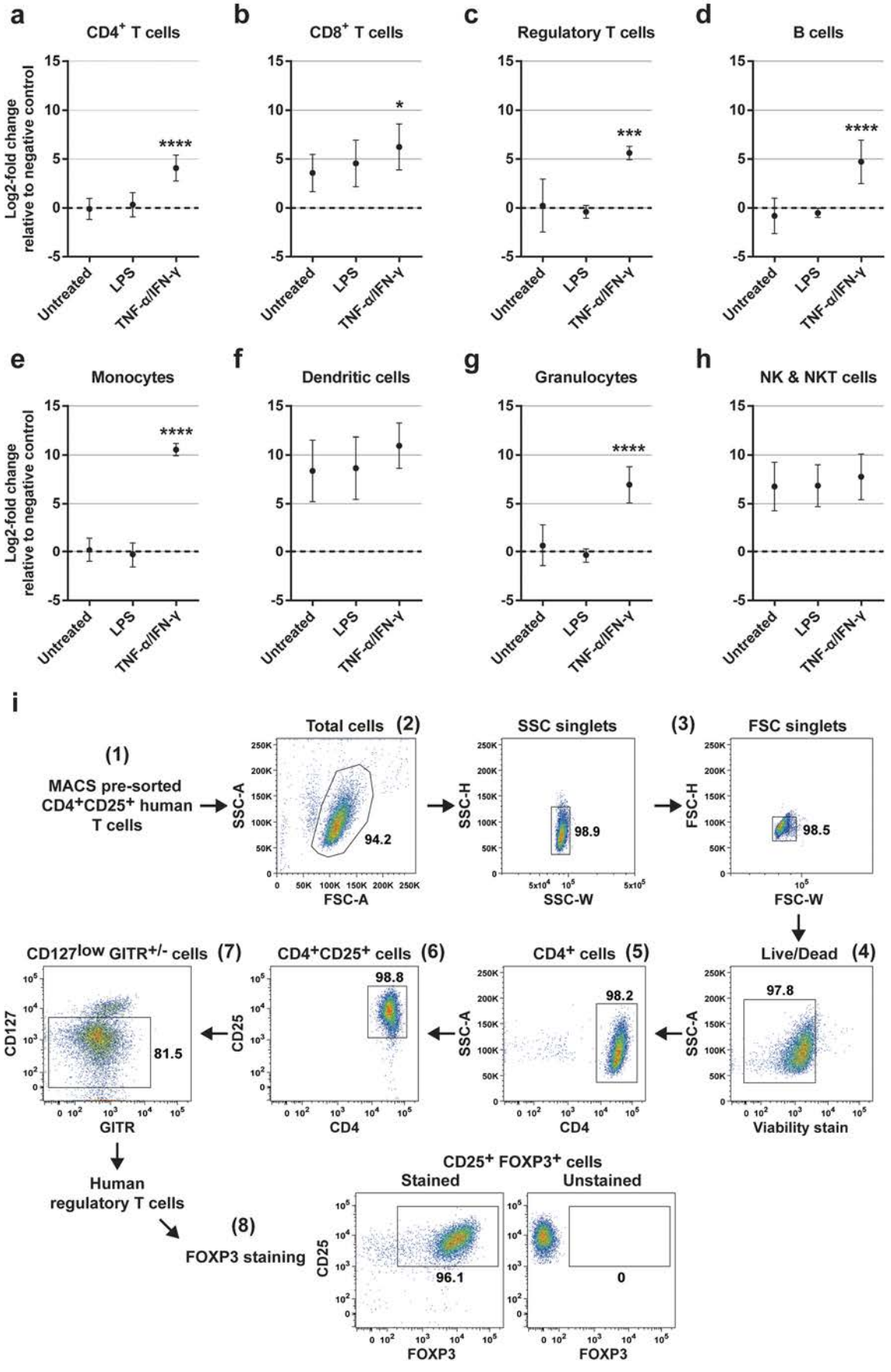
Statistical analysis

For statistical analysis, we used the GraphPad Prism 6 software (version 6.04) (GraphPad Software, Inc., USA). Statistical significance was determined by applying an ordinary one-way ANOVA followed by a multiple comparison of the untreated sample with each of the treated samples. The multiple comparison was analysed by the uncorrected Fisher's LSD test.

Results

To determine in which of the main leukocyte populations FAT10 is expressed or inducible by different stimuli, the different leukocyte subsets were isolated from human

Fig. 1 *FAT10* mRNA expression in different human leukocyte populations. The single immune cell subsets were isolated by magnetic (MACS) (**a**, **b**, **d**–**h**) or fluorescence-activated cell sorting (FACS) (**c**) from the PBMC or PMNC fraction of peripheral blood after density centrifugation. The isolated cells were left untreated, treated with 1 μg/mL LPS, or 400 U/mL TNF and 200 U/mL IFN-γ for 24 h. Subsequently, *FAT10* mRNA expression levels were determined by quantitative real-time RT-PCR. Expression levels in each sample were normalised to *RPL13a*, *SDHA*, and *TBP*. We excluded *TBP* as a reference gene for granulocytes, dendritic cells, and regulatory T cells (Treg) due to its unstable expression. Finally, we calculated the log2-fold change of expression relative to unstimulated HEK293T cells as negative control (depicted as dashed line). For sorting of human Tregs, the following strategy was applied (**i**). MACS pre-sorted human Tregs were stained for CD4, CD25, CD127, GITR, and viability, and subjected to FACS (1). First, total cells were identified (2), followed by exclusion of doublets (3) and dead cells (4). Then, cells were gated on $CD4^+$ cells (5), followed by gating on $CD4^+$ $CD25^+$ cells (6). Finally, human Tregs were sorted from the gate including $CD127^{low}$ $GITR^{+/-}$ cells. To assess successful sorting, Tregs were stained for intracellular Foxp3 for re-analysis (8). Graphs display the mean ± SD of three to six independent experiments ($n = 3$ (Treg); $n = 4$ ($CD14^+$, $CD15^+$, PanDC); $n = 5$ ($CD4^+$, $CD56^+$); $n = 6$ ($CD8^+$, $CD19^+$)). Numbers next to gates indicate percentage of positive cells of parent populations. To determine statistical significance, an ordinary one-way ANOVA followed by the uncorrected Fisher's LSD test was applied only comparing untreated with treated samples. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$; SSC, side scatter; FSC, forward scatter; A, area; W, width; H, height


a
CD4+ T cells
b
CD8+ T cells
c
Regulatory T cells
d
B cells
e
Monocytes
f
Dendritic cells
g
Granulocytes
h
NK & NKT cells
Log2-fold change relative to negative control
Untreated
LPS
TNF-α/IFN-γ
i
(1)
MACS pre-sorted CD4+CD25+ human T cells
Total cells (2)
SSC singlets
(3)
FSC singlets
Live/Dead (4)
CD4+ cells (5)
CD4+CD25+ cells (6)
CD127low GITR+/- cells (7)
Human regulatory T cells
(8)
FOXP3 staining
CD25+ FOXP3+ cells
Stained
Unstained
94.2
98.9
98.5
97.8
98.2
98.8
81.5
96.1
0

peripheral blood or murine spleen via magnetic sorting (MACS) or in case of regulatory T cells by FACS (Figs. 1i and 2h). The purity of the cell preparations was assessed by flow cytometric re-analysis and varied between 75.5 and 100% (Tables 5 and 6). The purified cells were left untreated, stimulated overnight with IFN-γ and TNF, or with LPS. Finally, we assessed human and murine *FAT10* expression by qPCR analysis (Figs. 1 and 2) and analysed human FAT10 protein level in bulk leukocytes by western blot analysis after immunoprecipitation (Fig. 3).

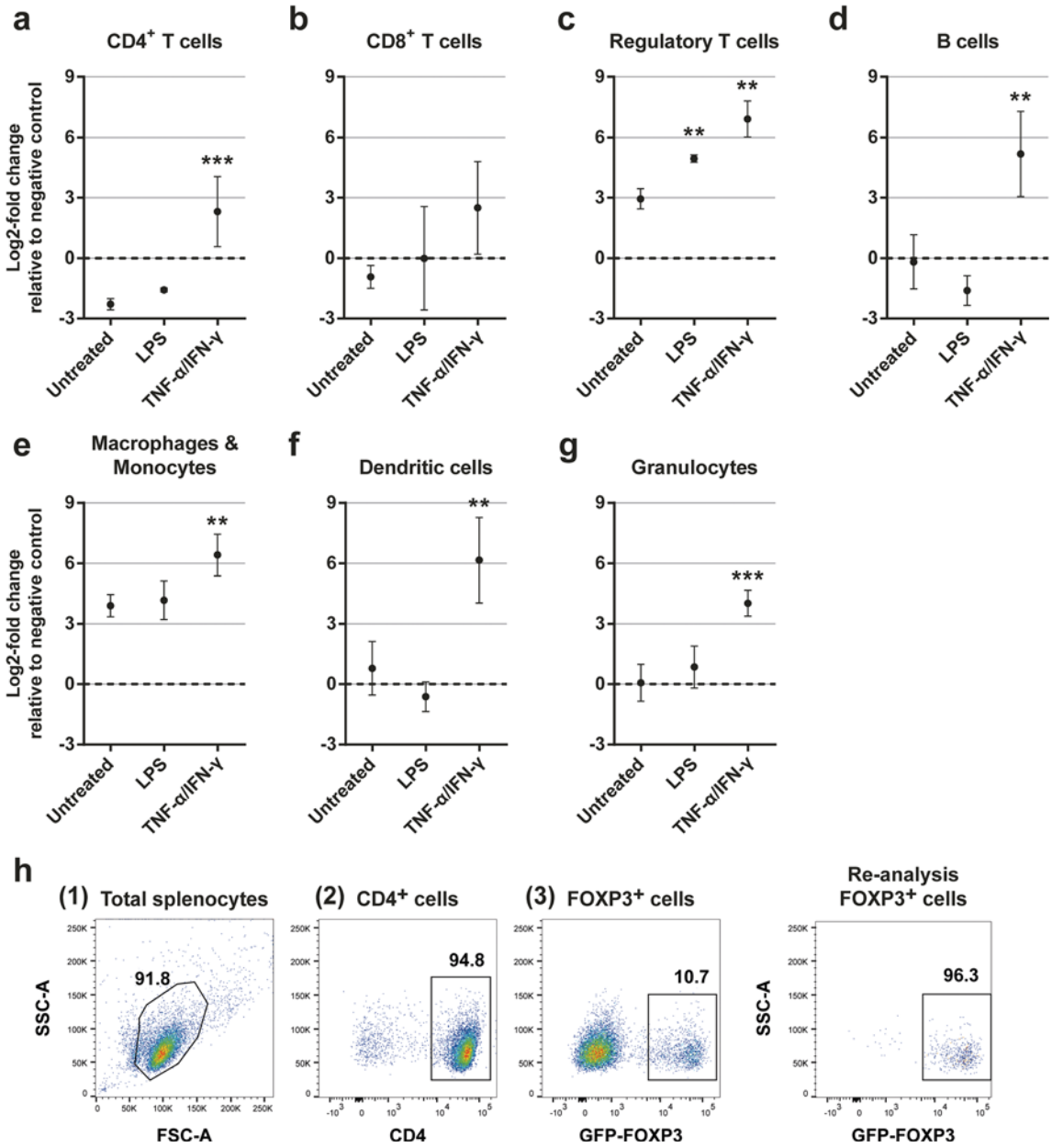


Fig. 2 *Fat10* mRNA expression profile of immune cell subsets from mouse spleen. Murine cells were sorted magnetically (**a**, **b**, **d**–**g**) or were purified by FACS (**c**) from spleens of C57BL/6 mice. After isolation, the cells were stimulated with 1 μg/mL LPS or 400 U/mL TNF and 200 U/mL IFN-γ for 24 h or were left untreated. Then, *Fat10* mRNA expression was quantified by real-time RT-PCR. We used cDNA prepared from total splenocytes of FAT10-deficient mice as negative control (shown as dashed line). We depicted *Fat10* expression as log2-fold change normalised to *Rpl13a* and *Actb*. **h** For isolation of mouse Tregs, the following steps were performed: (1), total splenocytes from C57BL/6 Foxp3-GFP reporter mice were gated. Then, cells were gated on $CD4^+$ cells (2), followed by GFP^+ cells (3). The sorting efficiency was evaluated by the percentage of GFP^+ cells from the sorted population (re-analysis). Graphs show the mean ± SD of three independent experiments, except for regulatory T cells where two experiments with similar outcome were performed. Numbers next to gates indicate percentage of positive cells of parent populations. Statistical significance was determined with an ordinary one-way ANOVA followed by the uncorrected Fisher's LSD test only comparing untreated with treated samples. $^{**}p < 0.01$, $^{***}p < 0.001$; SSC, side scatter; FSC, forward scatter; A, area

Table 5 Purity of the isolated human immune cell subsets

Cell type	Marker	Mean ± SD
$CD4^+$ T cells	CD4,CD3	90.2 ± 8.2
$CD8^+$ T cells	CD8,CD3	84.8 ± 4.6
NK and NKT cells	CD56,CD3	82.6 ± 2.9
Monocytes	CD14	87.3 ± 10.1
Granulocytes	CD15	90.1 ± 4.6
B cells	CD19	89.5 ± 6.7
Dendritic cells	HLA-DR,CD303	75.7 ± 6.0
Regulatory T cells	CD25,Foxp3	92.6 ± 2.3

Expression profile of *FAT10* in human leukocyte populations

In cells of the adaptive immune system, namely $CD4^+$ T cells, B cells, and Treg, *FAT10* mRNA was expressed neither in the untreated state nor after induction with LPS (Fig. 1a, c, d). Interestingly, $CD8^+$ T cells expressed *FAT10* already when not treated, and induction with LPS increased expression of *FAT10* slightly but not significantly (Fig. 1b). Induction with the pro-inflammatory cytokines IFN-γ and TNF significantly increased *FAT10* mRNA levels in $CD4^+$ and $CD8^+$ T cells, Treg, and B cells (Fig. 1a–d). The observed expression levels, however, were similar between the cell types ranging from 4.1 to 6.2 log2-fold change meaning that the increase of *FAT10* expression under inflammatory conditions was similar between these cell types regardless of the basal expression.

Next, we analysed the expression of *FAT10* in cells of the innate immune system (Fig. 1e–h). Monocytes and granulocytes showed no *FAT10* expression when untreated and stimulated with LPS (Fig. 1e, g). This is in line with a former report showing no expression of *FAT10* in unstimulated monocytes isolated freshly from PBMCs and granulocytes derived from $CD34^+$ haematopoietic progenitors (Bates et al. 1997). Of all human cell types examined, DC expressed the highest levels of *FAT10* already when untreated, which did not increase further upon induction with LPS (Fig. 1f). So far, *FAT10* expression in DC was reported for in vitro-generated, mature DC from

Table 6 Purity of the purified murine leukocytes

Cell type	Marker	Mean ± SD
$CD4^+$ T cells	CD4	86.2 ± 0.9
$CD8^+$ T cells	CD8	93.3 ± 0.9
Macrophages and monocytes	CD11b	92.8 ± 1.3
Granulocytes	Ly-6G	93.1 ± 1.0
B cells	CD19	87.6 ± 1.4
Dendritic cells	CD11c	96.3 ± 1.2
Regulatory T cells	Foxp3-GFP	100.0 ± 0.0

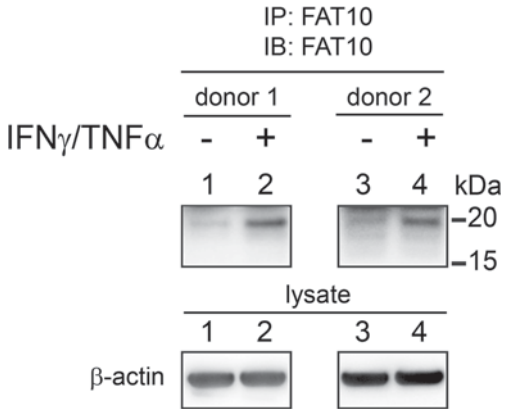


Fig. 3 Detection of endogenous FAT10 protein expression in cytokine-stimulated and unstimulated human PBMCs. Peripheral blood mononuclear cells (PBMCs, 4×10^8) from two healthy donors were cultivated in medium, supplemented with IFN-γ and TNF to induce endogenous FAT10 expression, or were left untreated, as indicated. After 30 h of incubation, cells were harvested, lysed, and subjected to immunoprecipitation using a monoclonal FAT10-reactive antibody (4F1, reference (Aichem et al. 2010)). Endogenous FAT10 in immunoprecipitates was detected by western blotting, using a polyclonal FAT10-reactive antibody (Hipp et al. 2005). β-Actin in total lysates prior to immunoprecipitation was used as loading control

$CD34^+$ haematopoietic progenitors and $CD14^+$ monocytes which reflected $CD1a^+$ Langerhans-like DC and inflammatory DC, respectively (Bates et al. 1997; Lukasiak et al. 2008; Ebstein et al. 2009). Yet, the dendritic cell population investigated here consisted of plasmacytoid and myeloid DC, which shows that, despite the lack of inflammatory signals and maturation, DC have a high basal expression level of *FAT10* in vivo. Remarkably, untreated natural killer and natural killer T (NK and NKT) cells displayed high *FAT10* expression, which was not enhanced upon LPS stimulation (Fig. 1h). This basal expression increased marginally when the cells were treated with the pro-inflammatory cytokines. *FAT10* expression was most stable in NK and NKT cells irrespective of treatment ranging from 6.8 (untreated) to 7.8 log2-fold change (cytokine-treated). Pro-inflammatory cytokines up-regulated *FAT10* mRNA in monocytes and granulocytes significantly (Fig. 1e, g), whereas in DC it increased only marginally (Fig. 1f). Here, monocytes showed the most striking increase in *FAT10* expression (10.6 log2-fold change), which reached levels similar to DC (10.9 log2-fold change) (Fig. 1e, f) and were the highest expression levels observed upon cytokine induction. In cytokine-induced granulocytes, *FAT10* expression rose to levels (6.9 log2-fold change) similar to $CD8^+$ T cells (6.2 log2-fold change) (Fig. 1b, g).

Overall, these results suggest that human *FAT10* is not only expressed ubiquitously under inflammatory conditions but also cell type-specific in a non-inflammatory environment. Furthermore, $CD8^+$ T cells, DC, and NK

and NKT cells expressed *FAT10* in an untreated state, which only increased insignificantly upon IFN-γ and TNF treatment. This suggests that FAT10 performs functions specifically required in these circulating immune cell types in their naïve, immature, or memory state.

Fat10 expression pattern in murine immune cell subsets

In cells of the adaptive immune system such as $CD4^+$ and $CD8^+$ T cells as well as B cells, we did not detect *Fat10* expression in untreated or LPS-treated cells (Fig. 2a, b, d). Only the cytokines IFN-γ and TNF jointly induced expression of *Fat10* in these cell types. The mRNA levels were similar in $CD4^+$ and $CD8^+$ T cells that were 2.3 and 2.5 log2-fold change, respectively, but only increased significantly in $CD4^+$ T cells. In B cells, the level of *Fat10* expression was twice as high (5.2 log2-fold change). Notably, Treg showed basal *Fat10* expression, which rose significantly upon LPS treatment (Fig. 2c). When treated with cytokines, Treg expressed *Fat10* even stronger attaining levels of 6.9 log2-fold change. High basal expression of *Fat10* in Treg is in accordance with a previous report showing that overexpression of *Foxp3* leads to *FAT10* up-regulation (Ocklenburg et al. 2006). These results infer that all cells of the murine adaptive immune system express *Fat10* in an inflammatory setting since the pro-inflammatory cytokines IFN-γ and TNF induced its expression in the aforementioned cell types.

We also analysed murine granulocytes, macrophages and monocytes (M&M), and DC, i.e. cells of the innate immune system, for *Fat10* expression (Fig. 2e–g). DC showed low basal *Fat10* expression when untreated (Fig. 2f) and LPS treatment completely repressed this basal expression. In granulocytes, we did not detect *Fat10* mRNA in untreated cells, but could stimulate *Fat10* expression with LPS slightly (Fig. 2g). Both cell types up-regulated *Fat10* significantly upon cytokine induction (Fig. 2f, g), where DC expressed *Fat10* at 6.2 log2-fold change and granulocytes at 4.0 log2-fold change. Highest expression levels were found in M&M (Fig. 2e). These cells had high basal *Fat10* expression, which significantly increased with cytokines but not with LPS. When stimulated with the pro-inflammatory cytokines IFN-γ and TNF, up-regulation of *Fat10* mRNA expression in DC and M&M reached similar levels of 6.2 log2-fold change in DC and 6.4 log2-fold change in M&M (Fig. 2e, f).

Taken together, our results indicate that upon IFN-γ and TNF treatment, murine *Fat10* is expressed in all immune cell subsets, which we analysed. Interestingly, M&M as well as Treg already express *Fat10* in a naïve state or non-inflammatory setting suggesting that Fat10 is necessary for cell type-specific functions unrelated to acute inflammation.

FAT10 protein expression is up-regulated upon stimulation of human leukocytes with IFN-γ and TNF

To test whether FAT10 protein expression correlated with *FAT10* mRNA expression, human PBMCs were either left unstimulated or were stimulated for 30 h with IFN-γ and TNF in vitro before combined immunoprecipitation and western blot analysis was performed. FAT10 protein was weakly expressed in human leukocytes but was up-regulated upon cytokine stimulation indicating that *FAT10* mRNA and protein expression levels correlated in human leukocytes (Fig. 3).

Discussion

We have examined the expression levels of the cytokine-inducible ubiquitin-like modifier FAT10 in different subsets of primary immune cells. The purpose of this study was to generate an expression profile of FAT10 that can guide future FAT10 research and may help to avoid that cell type-specific functions are overlooked. Due to the limited numbers of the primary immune cell populations, which we could purify from human peripheral blood, and due to a lack of a sensitive antibody for murine FAT10, we have limited our study to the quantitative analysis of *FAT10* mRNA expression in purified human and murine immune cell populations and FAT10 protein expression in bulk human PBMCs. In bulk human leukocytes, FAT10 protein expression paralleled the low basal and prominent cytokine-inducible expression of human and mouse *FAT10* mRNA measured in the different cell types. Moreover, we have shown previously that *FAT10* mRNA and protein expression closely correlate (Aichem et al. 2012), probably because FAT10, serving as a proteasome degradation signal, is rapidly degraded by the proteasome with an invariant half-life of approximately 1 h in the cell types analysed to date (Hipp et al. 2005; Schmidtke et al. 2014). Therefore, it is reasonable to assume that the herein reported amounts of *FAT10* mRNA will very likely closely reflect FAT10 protein levels in immune cell populations.

Previously, it has been reported that overexpression of *Foxp3*, a specific marker of Treg, led to *FAT10* up-regulation and that *FAT10* expression was found in long-term cultures of $CD4^+CD25^{hi}$-derived human Treg (Ocklenburg et al. 2006). Ocklenburg and colleagues established long-term cultures of human Treg by TCR stimulation, IL-2 supplementation, and co-culture with irradiated Epstein-Barr virus (EBV)-transformed B cells. We, however, cultured Treg short term in the absence of said stimuli and did not detect *FAT10* expression in naïve human Treg. Yet, *FAT10* was inducible in human and murine Treg by cytokines indicating that Treg express *FAT10* transiently under inflammatory conditions. Interestingly, we found that murine $Foxp3^+$ Treg express *Fat10* in a naïve state,

which increased significantly upon LPS and cytokine stimulation. Thus, transient *FAT10* expression could be involved in fine-tuning Treg function during inflammation. Additionally, Ocklenburg and colleagues showed that *FAT10* overexpression in $CD4^+CD25^-$ T cells enhanced CD25 surface expression and reduced *IL-4* and *IL-5* mRNA levels, but also inhibited proliferation and reduced the Ca^{2+}-flux upon TCR stimulation (Ocklenburg et al. 2006). We found that human and murine $CD4^+$ T cells express FAT10 only in presence of pro-inflammatory cytokines. Therefore, it is conceivable that FAT10 influences the phenotype of $CD4^+$ T cells in the course of an immune response. Next, we examined human and murine B cells in which we did not detect basal expression of *FAT10*. The lack of *FAT10* expression in B cells was surprising as it is contrary to what has been described by Bates et al. (1997). They found that *FAT10* is highly expressed in most EBV-transformed mature B cell lines and not in precursors B cell lines. Consequently, *FAT10* expression appeared to be characteristic for mature B cells as its expression also occurred independent of EBV infection. Yet, not all mature B cell lines expressed *FAT10.* Hence, it cannot be ruled out that the observed *FAT10* expression was an effect of the EBV latency rather than B cell maturity because EBV latency causes NF-κB and Stat-3 activation (Laherty et al. 1992; Li and Bhaduri-McIntosh 2016), which again can lead to increased *FAT10* expression (Choi et al. 2014). In accordance with these results, we conclude from our data that non-transformed mature B cells do not express *FAT10* unless in an inflammatory setting in the presence of IFN-γ and TNF.

FAT10 is very likely involved in antigen presentation as has already been suggested by Bates and colleagues (Bates et al. 1997) and experimentally supported during studies in which FAT10 was fused N-terminally to viral antigens (Ebstein et al. 2012; Schliehe et al. 2012). Hence, it was of special interest to analyse FAT10 expression in professional antigen-presenting cells (APCs), such as B cells, DC, and M&M. We found that upon stimulation with pro-inflammatory cytokines, i.e. representing an inflammatory situation, *FAT10* was induced strongly in all APCs further supporting a role of FAT10 in antigen presentation. In fact, human DC representing myeloid and plasmacytoid DC, and murine M&M already showed high basal *FAT10* expression. Although this level of expression did not change upon LPS stimulation, it increased further in the presence of pro-inflammatory cytokines. It will be interesting to find out why these distinct APC subsets already express *FAT10* in the absence of inflammation and the other APC subtypes do not.

Another intriguing finding was that apart from human DC, human naïve $CD8^+$ T cells and naïve NK and NKT cells expressed *FAT10* at high basal levels. The expression level changed marginally upon LPS stimulation, and insignificantly increased upon cytokine stimulation. This finding suggests that FAT10 is very likely involved in functions other than antigen presentation. The potential functions that FAT10 might fulfil in these cell types may be exerted in all of them. Indeed, $CD8^+$ T cells and NK and NKT cells bear close resemblance to each other (Narni-Mancinelli et al. 2011; Sun and Lanier 2011). These similarities are, for example, that both cell types undergo an educational process, exert similar effector functions when activated, and are able to form an immune memory. We also cannot exclude that these potential new functions of FAT10 are restricted to the human immune system since we could not detect *Fat10* expression in murine $CD8^+$ T cells.

Noteworthily, we observed not only cell type-specific but also species-specific differences in basal *FAT10* expression. Most strikingly, antigen-presenting cells differed between human and mouse regarding *FAT10* expression. Human DC but not murine DC expressed high levels of *FAT10* and, on the contrary, *Fat10* expression was readily detectable in murine M&M but was absent in human monocytes. Similarly, murine Treg and human $CD8^+$ T cells showed basal *Fat10* expression whereas no expression was detected in their human and murine counterparts, respectively. On the one hand, these differences could point towards a differential expression pattern of *FAT10* in the different immune cell types depending on their tissue location, since we used peripheral blood as a source for human immune cell subsets and isolated leukocytes from mouse spleen. On the other hand, if the observed cell type-specific expression pattern is not tissue-dependent, the discrepancy between the human and murine immune system could suggests that functions of FAT10 might differ between species. Alternatively, this discrepancy might result from a different pathogen burden of humans as compared to mice kept in a specific pathogen-free environment.

Coming to know which leukocyte populations in humans and mice express FAT10, and to what extent its expression is inducible by LPS and the pro-inflammatory cytokines TNF and IFN-γ will avoid overlooking potential new functions of FAT10 relating to the immune system.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international and national guidelines for the care and use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution.

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