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Proteomic definition of human mucosal-associated invariant T cells determines their unique molecular effector phenotype

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Abbreviations: cCD8 T cell: conventional, non-MAIT CD8 T cell, MAIT cell: mucosal-associated invariant T cell, NK cell: natural killer cell, LAAO: L-amino-oxidase, IS: immunological synapse, MS: mass spectrometry, LC: liquid chromatography, log₂RF: log₂ regulation factor, iTRAQ: isobaric Tags for Relative and Absolute Quantitation, MR1: MHC-class I-related molecule 1, STED: Stimulated Emission Depletion

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Abstract

Mucosal-associated invariant T cells (MAIT) constitute the most abundant anti-bacterial CD8+ T cell population in humans. MR1/TCR-activated MAIT cells were reported to organize cytotoxic and innate-like responses but knowledge about their molecular effector phenotype is still fragmentary. Here, we have examined the functional inventory of human MAIT cells (CD3+Va7.2+CD161+) in comparison with those from conventional non-MAIT CD8+ T cells (cCD8+) and NK cells. Quantitative mass spectrometry characterized 5500 proteins of primary MAIT cells and identified 160 and 135 proteins that discriminate them from cCD8+ T cells and NK cells donor-independently. Most notably, MAIT cells showed a unique exocytosis machinery in parallel to a pro-inflammatory granzyme profile with high levels of the granzymes A, K and M. Furthermore, 24 proteins were identified with highest abundances in MAIT cells, including CD26, CD98 and L-amino-oxidase (LAAO). Among those, expression of granzyme K and CD98 were validated as MAIT-specific with respect to non-MAIT CD8+ effector subsets and LAAO was found to be recruited together with granzymes, perforin and CD107a at the immunological synapse of activated MAIT cells. In conclusion, this study complements knowledge on the molecular effector phenotype of MAIT cells and suggest novel immune regulatory functions as part of their cytotoxic responses.

Introduction

Mucosal-associated invariant T cells (MAIT cells) are the most abundant T cell subset with antimicrobial properties, constituting up to 50 % of T cells in the liver and 1-10 % of T cells in peripheral blood of humans [1–3]. In humans, MAIT cells are defined by the expression of a semi-invariant TCRα chain, Va7.2, associated with a restricted TCRβ repertoire [4],[5]. Furthermore, they show high expression of C-type lectin CD161, multi-drug resistant protein MDR1 and CD26 [6],[7]. With regard to co-receptors, the majority of MAIT cells are CD8αα, CD8αβ or double negative T cells, with only a small proportion expressing CD4 [8]. MAIT cells are restricted by MHC-class I-related molecule MR1, and are absent in MR1 knockout mice [9]. MR1 is the most conserved MHC class I or MHC class I-related molecule between mice and humans [10], and it presents small molecular ligands derived from bacterial riboflavin to MAIT cells [11],[12]. Consequently, only bacteria with a functional riboflavin pathway have been shown to activate MAIT cells in vitro [8]. Interestingly, MR1-dependent processes have been recognized as therapeutic target, as drugs were shown to interfere with MAIT cell activity [13].

CD8+ T cells can be classified into four subsets: naïve (Tn) central memory (Tcm), effector memory (Tem), which are predominantly but not exclusively MAIT cells, and TemRA cells. While Tem cells possess proliferative capacity and produce IL-2, the further differentiated Tem and TemRA cells are characterized by their ability to rapidly exert cytotoxic effector functions. [14] Unlike the conventional CD8+ T cell compartment (cCD8+), but like NK cells, all MAIT cells are innately pathogen-reactive and display a unique effector memory-like phenotype towards bacterial infections [15]. Upon recognition of the MR1:ligand complex they can readily produce pro-inflammatory cytokines IFN-γ, TNF-α and IL-17 [6],[16]. Most notably, MAIT cells have the capacity to directly lyse bacterially infected cells contact-dependently by forming an immunological synapse (IS). IS formation in other cytotoxic lymphocytes such as conventional CD8+ T cells and NK cells is accompanied by actin accumulation, the convergence of cytolytic granules towards the IS and their subsequent polarized...
release and fusion with the target cell [17]. These granules contain cell type-specific inventories of effector molecules to realize specific phenotypes. Until now, resting MAIT cells were validated to express perforin and granulysin [3], as well as granzymes A and K [18]. Furthermore, it was demonstrated that expression of granzyme B and perforin increases in activated MAIT cells, thereby enhancing their cytotoxic capacity [18] and it is known that cytolytic MAIT cells express high levels of the transcriptional regulators PLZF, RORγt, Eomes and Helios [19]. However, a systematic inspection of effector proteins in MAIT cells is missing, and their recruitment to the IS of MAIT cells is not investigated.

In this study, we aimed to define the protein network beyond the unique effector phenotype of MAIT cells. Thereby, the intention was not to segregate effector-memory MAIT cells specifically from non-MAIT effector-memory CD8\(^+\) T cells. Instead, we hypothesized that the protein network and processes contributing to the MAIT effector phenotype can be best recognized in relation to all conventional, non-MAIT CD8 T cells (cCD8\(^+\), including T\(_{Nc}\), T\(_{CM}\), T\(_{EM}\) and T\(_{EMRA}\)) and cytotoxic NK cells, and performed comparative proteome studies of these three populations. Quantitative mass spectrometry characterized the MAIT proteome in five healthy donors and identified MAIT-specific but donor-independent protein abundances. In comparison to NK and cCD8\(^+\) T cells, we found a notable adaptation of proteins involved in exocytosis indicating a unique effector machinery at the immunological synapse (IS) of MAIT cells. This was corroborated by the identification of a unique granzyme profile including pro-inflammatory granzymes A, K and M as well as LAAO that we here introduce as a novel component of the IS from anti-bacterial MAIT cells.

Results

Identification of the MAIT proteome

The first step of this study was the unambiguous definition of MAIT cells in preparation for proteomic analyses. We used CD3, CD161 and semi-invariant TCR chain Va7.2 to distinguish MAIT cells (CD3+CD161++Va7.2+; Error! Reference source not found.A) from other PBMCs in blood donations of healthy individuals. Secondly, we ensured the MAIT cell responsiveness to activating stimuli in these isolates by an anti-bacterial-assay. We then confronted PBMCs isolated from donor blood with THP-1 cells that had been fed with fixed E. coli overnight and thus have the potential to induce MAIT cell activation [20]. MAIT cells isolated from the blood of healthy donors respond as expected to the stimulus with surface expression of activation markers CD107a (Error! Reference source not found.B) and CD69 (Error! Reference source not found.C). This confirmed their resting and responsive state, and their suitability for proteomic analyses.

Next, we developed a quantitative proteomic approach, which should allow the identification of regulators and proteins defining the MAIT phenotype. Our main interest was to identify the effector components that distinguish MAIT cells from conventional cytotoxic immune cells on the protein level. We thus sorted MAIT cells as well as conventional, non-MAIT CD8 T cells (“cCD8\(^+\), CD3’CD8\(^-\)”) from the peripheral blood of five healthy human donors. In addition, and to be able to identify potential innate-like MAIT-specific processes that are not displayed in cCD8\(^+\) T cells, we also sorted innately cytotoxic NK cells (CD3’CD56\(^+\)) from the same donors (see gating strategy in Error! Reference source not found.). Proteomes of the three cytotoxic cell subsets were extracted donor-
Peptides in the CD8 subset in total MAIT (81%) cells resistant transporter (MDR1), α-α chain while the difference in CD8 cells was assessed. CD8 α chain is a marker of effector memory T cells, expressed by most MAIT cells. Furthermore, we could confirm CD26 and multidrug resistance (MDR1) as described as MAIT cell markers. Increased expression of CD44 and CD127 with already published data (MAIT: +1.70; CD8: +2.39). Indeed, NK cells can express this subunit of the CD3 complex (MAIT: +2.39; CD8: +1.28). We also detected CD16 to be highly abundant in NK cells (MAIT: +0.24; CD8: -0.24) as expected. CD3ε was identified with similar expression in MAIT cells and CD8 T cells (MAIT: +0.07; CD8: +1.70). Indeed, NK cells can express this subunit of the CD3 complex (MAIT: +0.24).Proteomic data could detect CD16 to be highly abundant in NK cells (MAIT: +2.88), where it is highly expressed. Furthermore, we could confirm CD26 and multidrug resistant transporter (MDR1), which both have been described as MAIT cell markers [6],[7] and whose high abundance in MAIT cells concurred with already published data (MAIT: +2.39; MDR1 +1.07). We also detected increased expression of CD44 and CD127 in MAIT cells (MAIT: +0.65; CD127 +0.93), which are markers for effector memory T cells [6]. CD127 has already been shown to be highly expressed in MAIT cells [27]. These data further underline the effector memory phenotype of MAIT cells. Thus, data of “proof of concept” proteins and MAIT cell markers was found in full accordance with literature and reflects the effector memory phenotype of MAIT cells.

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We then aimed to identify potential upstream regulators that might be responsible for the differential phenotype of MAIT cells. We used the quantitative information (median log_{2}RF MAIT:cCD8⁺) of all 5500 identified proteins to perform an upstream network analysis by Ingenuity Pathway Analysis (IPA). Upstream network analysis resulted in a list of ten potential upstream regulators (Supporting Information Table 3), which are able to contribute for the protein abundances in our MAIT cells. Importantly, upstream analysis did not point to involvement of transcription factors PLZF, RORγt, Eomes and Helios, which are highly expressed in MAIT cells [19]. Rather, in silico analyses by IPA suggest novel factors that potentially play a role in the molecular effector phenotype of MAIT cells. Among them were transcription factors ERG, SATB1 and SirT1 (SIRT1), surface receptors like interferon alpha/beta receptor 2 (IFNAR2) and integrin alpha-L (ITGAL) as well as cytokines C-C motif chemokine 5 (CCL5, RANTES) and interleukin 8 (CXCL8). Of those, integrin alpha-L actually was found with increased abundance in MAIT cells as compared to cCD8⁺ T cells (median log_{2}RF MAIT:cCD8⁺ 0.63).

To further evaluate the differences between MAIT cells, cCD8⁺ T cells and NK cells, we next used two criteria that allowed the definition of donor-independently regulated proteins: (i) we only examined the 2619 proteins that were robustly identified in all five donors (Error! Reference source not found.), (ii) we considered only those proteins as regulated that were found consistently regulated and match the significance criteria again in all five donors. For that, we applied an established statistical method to calculate the probability that the log_{2}RF of a protein can be explained by variation [28]. A protein was only considered to be significantly regulated if this probability was lower than 5% for all five donors. This strategy confirmed all proof of concept proteins introduced before (Error! Reference source not found.) and defined a distinct set of subset-specific proteins. In total, 160 proteins were found to discriminate MAIT cells and cCD8⁺ T cells in all five donors (Supporting Information Table 4 and Supporting Information Table 5), thereby 110 proteins were found to be significantly upregulated and 50 downregulated in MAIT cells. Likewise, the comparison of MAIT cells and NK cells revealed 135 proteins to be differentially abundant, with 77 proteins determined as upregulated and 58 proteins as downregulated in MAIT cells.

In conclusion, we developed a sensitive quantitative proteomics approach that allows us to robustly identify donor-independently regulated effector components. Furthermore, we could show that our proteomic data for already published markers are in accordance with literature and suggest a new set of potential regulators for MAIT cell proteins.

**MAIT cells have an optimized capacity for IS-mediated exocytosis of effectors**

The majority of MAIT cells are CD8⁺, but it is unclear which factors enable them to realize their innate-like effector phenotype. To address this, we first investigated which components discriminate MAIT cells from conventional CD8⁺ T cells. We hypothesized that proteins which mechanistically realize anti-bacterial effector responses are significantly abundant in MAIT cells and display the highest fold change in comparison to cCD8⁺ T cells. Focusing on the top-upregulated proteins with the highest median log_{2}RF (Supporting Information Table 6) confirmed the importance of CD26 (DPP4), which was recently identified as a MAIT cell marker, and in parallel revealed six distinct components involved in exocytosis: Secernin-1 (median log_{2}RF MAIT:cCD8⁺ 1.07) has been shown to increase both the extent of secretion in mast cells, and the sensitivity of mast cells to stimulation.
with calcium [29]. Alpha-actinin 4 (MAIT:cCD8⁺ 1.07) is a calcium-dependent actin-binding protein mediating interactions between the membrane and actin filaments [30]. Unconventional myosins like myosin-if (MAIT:cCD8⁺ 1.39) are suspected to share similar properties to myosin, which is an actin-dependent motor protein [31]. Synaptotagmin-like protein 2 (Slp2, MAIT:cCD8⁺ 0.96) contributes to secretory lysosome exocytosis from cytotoxic lymphocytes and has been shown to be associated with the immunological synapse [32]. Cathepsin W (MAIT:cCD8⁺ 0.89) has been described to be expressed in effector CD8⁺ T cells and is secreted during target cell killing [33]. Finally, calcium-binding protein S100A4 was also identified to be significantly more abundant in MAIT cells, in comparison to cCD8⁺ T cells (MAIT:cCD8⁺ 1.43). We recently related high expression of S100A4 to the cytotoxic phenotype of CD56dim NK cells and demonstrated its recruitment to the immunological synapse upon target cell contact [34]. Importantly, MAIT cells show even higher abundance of S100A4 than NK cells (MAIT:NK 1.06). In summary, MAIT cells express a variety of components that promote exocytosis in a calcium-dependent manner, and might thus be able to secrete large amounts of granules rapidly after activation.

**MAIT cells show a unique pattern of effector molecules**

We next evaluated whether the innate and anti-bacterial phenotype of MAIT cells requires a specific adaptation of known immunologically active effectors proteins like perforin. Again, we compared MAIT cells with cCD8⁺ T cells but as before included data from NK cells, to allow the detection of functions that are not displayed in cCD8⁺ T cells. Interestingly, all important immunological effector molecules, i.e. perforin, all five human granzymes (A, B, H, K, M) and granulysin were found with subset-specific profiles in MAIT cells, NK cells and cCD8⁺ T cells (Error! Reference source not found.). Perforin has been observed as low abundant in resting MAIT cells [18], and proteomic data confirmed equal amounts of perforin as in cCD8⁺ T cells (MAIT:cCD8⁺ 0.26). In contrast, granzyme A was more abundant in MAIT cells, when compared with cCD8⁺ cells (median log₂RF MAIT:cCD8⁺ 1.60, MAIT:NK -0.08), while granzyme B was equally abundant in MAIT cells and cCD8⁺ T cells (MAIT:cCD8⁺ -0.05) and showed highest abundance in NK cells (MAIT:NK -2.08). Granzyme H was also highest expressed in NK cells, but showed significant regulation between MAIT cells and cCD8⁺ T cells also, being downregulated in MAIT cells (MAIT:cCD8⁺ -0.71, MAIT:NK -2.44). Granzyme K was highly abundant in MAIT cells, and identically expressed in NK and cCD8⁺ T cells (MAIT:cCD8⁺ 1.91, MAIT:NK 1.66). Granzyme H showed lower abundance in MAIT cells than in NK or cCD8⁺ T cells (MAIT:cCD8⁺ -0.71, MAIT:NK -2.44). Interestingly, granzyme M showed higher expression in MAIT cells than in cCD8⁺ T cells (MAIT:cCD8⁺ 0.92, MAIT:NK -0.84). Granulysin abundance in MAIT cells was lower than in NK cells, and similar to cCD8⁺ T cells (MAIT:cCD8⁺ 0.28, MAIT:NK -1.82).

Taken together, this study identified a unique profile of granzymes in MAIT cells, depending on highly expressed granzymes A, K and M, which are expressed in larger amounts than in cCD8⁺ T cells. Cytolytic proteins granzyme B and perforin were also identified, albeit in low amounts. Since granzymes A and K seem to play a pivotal role for the MAIT cell phenotype, we asked the question whether the high expression of these proteins is specific for MAIT cells, or representative for effector memory CD8 T cells in general. We checked granzyme A and K expression in MAIT cells by flow cytometry, and compared their abundance to naïve (TNa, CD45RA⁺CCR7⁺), central memory (TCEM, CD45RA⁻CCR7⁻) effector memory (TEM, CD45RA⁺CCR7⁻) and TEMRA (CD45RA⁺CCR7⁻) CD8 T cells, that are not MAITs (gating strategy see Supporting Information Figure 4). While granzyme A was found also
highly expressed in T_{EM} and T_{EMRA} cells (Error! Reference source not found.C), high expression of granzyme K was indeed detected only in MAIT cells and no other subset indicating its specific role in the MAIT effector phenotype (Error! Reference source not found.D).

**MAIT-specific proteins suggest LAAO as a lysosomal effector molecule**

We identified a unique granzyme pattern and superior exocytosis machinery of MAIT cells and thus wondered if this is corroborated by highly abundant protein functions in MAIT cells. Among the 160 significantly regulated proteins, we could identify a set of 24 proteins that revealed superior expression levels in MAIT cells of all donors, when compared with both cCD8\(^+\) T cells and NK cells (Table 1). These protein functions are most likely of importance for the MAIT phenotype, but in part might play a role in other effector subsets as well. CD26 can be found in this list, as well as granzyme K, which we proved before as MAIT-specific and the other exocytosis-promoting proteins mentioned above. We then investigated which other processes are controlled by those highly expressed proteins in MAIT cells. Strikingly, we identified a group of proteins in this list that is associated with proliferative control. Our proteomic data showed MAIT-exclusive upregulation of proteins L-amino-acid oxidase (LAAO or OXLA, median log\(_2\)RF MAIT:cCD8\(^+\) 2.00), galectin-3 (MAIT:cCD8\(^+\) 1.49) and both subunits of the CD98 heterodimer (MAIT:cCD8\(^+\) 2.38/1.14). CD98 is an amino acid transporter involved in the uptake of nutrients, and its loss results in cell death [35]. Flow cytometric analyses confirmed highly specific expression of CD98 on the surface of MAIT cells when compared with T_{Nv}, T_{CM}, T_{EM} and T_{EMRA} CD8 T cells (Supporting information Figure 5). Galectin-3 has been shown to be involved in the prevention and induction of apoptosis in T cells [36]. LAAO has been shown to inhibit the proliferation in T cells [37]. Interestingly, it was also suggested that LAAO is localized in lysosomal compartments and can be secreted by APCs [38]–[40]. Its high expression in MAIT cells prompted us to ask, if it plays a role in immune synapse (IS) formation or target cell killing.

**LAAO is recruited to the immunological synapse of MAIT cells**

After characterizing highly expressed effector molecules in MAIT cells, we wanted to characterize the MAIT cell immunological synapse (IS). Live cell imaging data has already shown contact-dependent killing of target cells, but to our knowledge the MAIT cell IS has not been investigated in detail. First, we wanted to clarify if the identified effector proteins indeed are recruited to the MAIT IS and thus support cytotoxicity. For that, we used a MAIT activation assay suitable for microscopic imaging, where THP-1 cells were fed with fixed *E. coli* BL21 overnight, and co-incubated with sorted MAIT cells for 30 minutes. Cells were then fixed, stained with different antibodies and THP-1:MAIT conjugates were analyzed by Stimulated Emission Depletion (STED) Microscopy. We stained for CD107a as lysosomal marker for secretory granules, and for effector molecules granzyme B, granzyme M and perforin. Importantly, we could observe granzymes M and B in secretory granules of MAIT cells, as both proteins colocalize with lysosomal marker CD107a (Error! Reference source not found.A, B). This confirmed the presence of these granzymes in MAIT cells whereby their localization in cytolytic granules underlined their importance for the effector phenotype of MAIT cells. Furthermore, we wanted to investigate if the highly expressed LAAO is indeed located in vesicles in MAIT cells, as well and whether these are recruited toward the IS upon MAIT activation. We observed the partial localization with cytolytic perforin (Error! Reference source not found.C)
and lysosomal marker CD107a (Error! Reference source not found.), and could confirm their recruitment to immunological synapse (Error! Reference source not found.). It is therefore tempting to speculate that LAAO is indeed secreted toward the MAIT target cell, but the specific purpose of this mechanisms needs further investigation. Taken together, we here present the first visualization of the MAIT cell immunological synapse, confirmed the IS-recruitment of perforin, granzymes B & M and suggest a role of LAAO in the effector phenotype of MAIT cells.

Discussion

So far, flow cytometric analyses of selected effector molecule expression in MAIT cells have been published [6],[18]. In 2011, flow cytometric analyses revealed multidrug resistance protein 1 (MDR1) to be prominently expressed in MAIT cells, and already reported high, MAIT-specific expression of CD26 [6], which has recently be suggested as a specific marker for MAIT cells [7]. Also, low expression of CD62L in MAIT was reported, which is consistent with their classification as effector cells. These results were now corroborated by donor-specific proteome analyses of ex vivo MAIT cells, which additionally identified effector memory marker CD44 to be highly expressed in MAIT cells (Error! Reference source not found.).

Regarding other approaches at the systems level, CD161\textsuperscript{hi} have been compared to CD161\textsuperscript{lo} cells within the T\textsubscript{EM} subset on the level of mRNA [41], while another transcriptome study comparatively analyzed CD8\textsuperscript{+}CD161\textsuperscript{+}, which largely overlap with MAIT cells, and CD8\textsuperscript{+}CD161\textsuperscript{+} T cells [42]. Importantly, proteomics complements knowledge from these transcriptome studies and gene by gene comparison indicated only very few (CD161\textsuperscript{hi}/CD161\textsuperscript{lo} T\textsubscript{EM}) or no (CD8\textsuperscript{+}CD161\textsuperscript{+}/CD8\textsuperscript{+}CD161\textsuperscript{+}) counter-regulations, e.g. a downregulated gene and the upregulation of the corresponding protein or vice versa (see Supporting Information Table 7). Thus, the majority but not all information provided by those transcriptome studies can indicate the corresponding protein level in MAIT cells. Interestingly, we found granzyme A among the genes, which according to transcriptome data from the T\textsubscript{EM} study are not in accordance with their abundance at the protein level. Since a high granzyme A protein level in MAIT cells was validated both by MS and flow cytometry, it appears likely that the abundance of granzyme A, and similar cases, is widely under post-transcriptional control in MAIT cells.

Notably, it could be shown by proteomics that MAIT cells also express granzyme M, although somewhat lower than NK cells (Error! Reference source not found.). Granzyme M is highly expressed in NK and NKT cells, and expressed in a third of all CD8\textsuperscript{+} T cells [43]. It was therefore been attributed a role in innate immunity [44], although more recent studies have revealed high expression of granzyme M in differentiated effector T cells [45]. The immune-regulatory roles of granzyme M at present are under debate [46]: Beside of its contribution to target cell killing [47]–[49], more recent studies indicate the importance of granzyme M in promoting inflammatory processes: In mice, granzyme M synergistically enhances the inflammatory response to LPS challenging [50], and furthermore promotes secretion of macrophage inflammatory protein-1 alpha (MIP-1\textalpha) from NK cells [51]. Interestingly, also highly expressed granzymes A and K have recently be described to show strong pro-inflammatory effects in reaction to bacterial stimuli [52]. Thus, granzyme M is the third rather pro-inflammatory granzyme with anti-bacterial properties that is highly expressed in MAIT cells. This provides further evidence for the phenotype of resting MAIT...
cells, which is apparently less dependent on cytotoxic effector proteins but rather on pro-inflammatory ones.

Based on quantitative information of 5500 proteins, we were also able to identify potential upstream regulators in silico that can explain differential protein abundances in MAIT cells and cCD8\(^+\) T cells (Supporting Information Table 3). Surprisingly, data analysis did not suggest any of the transcription factors that were reported as highly expressed in cytolytic MAIT cells (PLZF, RORyt, Eomes and Helios) [19]. Instead, reverse network analyses by Ingenuity Pathway Analysis indicated further upstream regulators. Among them were transcription factors ERG, SATB1 and Sirtuin-1, which have not been associated with MAIT cells before. Proteomic data indeed confirmed expression of SATB1 and Sirtuin-1 in MAIT cells. SATB1 has been implicated to play a role in T cell development, especially of regulatory T cells and NKT cells [53]. Sirtuin-1 modulates ROR\(\gamma\)T and drives Th17 cell generation [54]. Data analysis also suggested surface proteins (IFNAR, ITGAL) and cytokines (CCL5, CXCL8/IL-8). None of these proteins have been discussed in the context of MAIT cell immunology before. However, we were able to detect ITGAL in four donors (median log\(\_\)RF MAIT:cCD8\(^+\) 0.63) and statistics determined it to be regulated in three. Furthermore, MAIT cells exhibit high expression of CCR5, the receptor for CCL5 [6], which might further underline the importance of this cytokine for MAIT cell biology.

It is an interesting observation, that effector components promoting exocytosis processes are more abundant in MAIT cells than in cCD8\(^+\) T cells (Table 1). Most of these proteins are showing calcium-dependent activity, indicating a role during immune cell activation. Among those factors are secernin-1, myosin-1f, synaptotagmin-like protein 2, cathepsin W and alpha-actinin 4. Secernin-1 is not only upregulated when compared with cCD8\(^+\) T cells, like the others, but also when compared with NK cells (Table 1). Therefore, it seems to play an important role for the MAIT cell phenotype. Secernin-1 has been shown to promote exocytosis in mast cells in a calcium-dependent manner and increases their sensitivity towards calcium signaling, [29] and might also realize the same function in MAIT cells. Secernin-1 can additionally be secreted, and it is tempting to speculate that this is also the case in MAIT cells. Taken together, several proteins that are associated with the positive regulation of exocytosis or immune cell activation show high abundance in MAIT cells, and are typical for the MAIT cell phenotype. However, due to the heterogeneity of the exact functions of these proteins, the implications of their upregulation are not obvious. One possibility would be that exocytosis in MAIT cell is differently regulated and executed than in NK and cCD8\(^+\) T cells. When taking into account that MAIT cells display an innate-like phenotype and are therefore part of one of the first lines of defense against microorganisms, it is possible that they need to release their granules quicker and in larger amounts. Increased abundance of proteins like secernin-1, myosin-1f or synaptotagmin-like protein 2 could enable them to do so.

In comparison to cCD8\(^+\) and NK cells this study results in the identification of 24 protein functions that showed a superior abundance in MAIT cells (Table 1). Whereas granzyme K and CD98 could be already validated as MAIT-specific, other components, as demonstrated for granzyme A, seem to be of general importance for T cell effector subsets (Figure 3 C). Thus, those identified proteins have to be further validated not only in MAIT cells but additionally in effector memory (T<sub>EM</sub>) and T<sub>EMRA</sub> cCD8\(^+\) T cells. Furthermore, a dedicated membrane proteome approach might further complement this candidate list, since our total proteome approach could not detect known MAIT surface proteins, such as CD161 or IL18R in all donor samples.
With respect to their role in MAIT cells we noticed several molecules associated with the regulation of proliferation. It has already been suggested that MAIT cells die in chronic viral infection, due to activation induced cell death [16],[53]. Also patients with bacterial infections like *M. tuberculosis* infection showed decreased numbers of MAIT cells [8],[54], an observation that directly correlates with the increased susceptibility for secondary infections in critically ill patients [55]. MAIT frequency might thus be a determinant for protection, but the underlying regulatory components are incompletely defined, although it has been reported that MAIT cells are prone to apoptosis through elevated levels of caspases-3 and -7 [56]. Our proteomic data showed that proteins galectin-3, both parts of the CD98 heterodimer and L-amino-acid oxidase (LAAO) are highly upregulated in MAIT cells (Table 1). Galectin-3 is usually present in the cytoplasm but can be secreted by macrophage cell lines [57]. Interestingly, intracellular galectin-3 prevents apoptosis, while extracellular galectin-3 can induce cell death specifically in T cells [35]. Although the concentration needed for apoptosis induction is relatively high, it has been suggested that galectin-3 might increase its apoptotic capacity when expressed on the cell surface, similar to galectin-1 [58]. Although proteomic data indicated high abundance of galectin-3 in MAIT cells, localization could not be determined. It is possible that galectin-3 is relocated to the surface after certain stimuli or activation and there induces cell death in either target cells, or even other MAIT cells. The latter might be another method of a negative feedback mechanism to regulate MAIT cell activity in vivo. CD98, or LAT1, is a heterodimer that forms a transporter for large amino acids [59]. It has been reported that CD98 plays an important role in activated T cells by catalyzing the influx of amino acids that serve as nutrients [34]. The high abundance of CD98, which we could show to be specific for MAIT cells and not for other effector T cell subsets, implicates a state of readiness, which might be corroborated by observed upregulation of their exocytosis machinery. In this line we could characterize the dynamic formation of the MAIT cell immunological synapse demonstrating the recruitment of effector components (Error! Reference source not found.). After activation with the monocytic cell line THP-1 and bacteria, MAIT cells showed typical polarization and localization of granules towards the interface between MAIT cell and target cell. This process is typical for T cells [60]. We could also show that MAIT cells store the granzymes B and M in CD107a+ granules. Interestingly, microscopy data indicated a partial localization of L-amino-oxidase (LAAO) and CD107a, suggesting its presence in granules that are secreted into the target cell. As a major component of snake venom, this enzyme has been described to display cytotoxic and antibacterial properties through the production of H₂O₂ [61], and might also be a new factor in MAIT cell cytotoxicity. Furthermore, LAAO has been described to negatively regulated MAIT cell proliferation. It oxidizes L-amino acids with the consumption of FAD, and the thereby produces H₂O₂. It has been shown to be highly expressed in Th17 cells and even higher in CD4+CD161+ T cells [36]. Here, LAAO expression decreases IL-2 production and proliferation, and knock-down of LAAO-mRNA accordingly increased the proliferative capacity. Additionally, its expression is controlled by transcription factor RORyt [36], which is a master transcription factor for CD8+CD161++ T cells [16]. In MAIT cells, the pronounced abundance of LAAO has not been discussed yet. However, it is logical that the high expression of RORyt in MAIT cells [62] would also result in high expression of LAAO. Also in MAIT cells, this protein might inhibit proliferation, and lead to a similar, anti-proliferative phenotype as in Th17 cells.

In summary, this study complements knowledge about the molecular effector phenotype of MAIT cells. Their anti-bacterial capacity is donor-independently associated with proteins that mainly control exocytosis and proliferation, a unique granzyme effector composition and likely involves
LAAO that we suggest as a novel component of MAIT cell lytic granules. Based on the proteome of primary human MAIT cells it is tempting to speculate that this abundant T cell subset has notable immune-regulatory capacities in addition to its cytotoxic properties.

**Material and Methods**

**Cell culture**

This study was conducted in accordance with the rules of the Regional Ethics Committee of Lower Saxony, Germany and the declaration of Helsinki. Buffy coats from blood donations of healthy human volunteers who provided informed consent were obtained from the Institute for Clinical Transfusion Medicine, Klinikum Braunschweig, Germany. Blood donors' health is rigorously checked before being admitted for blood donation. This process included a national standardized questionnaire with health questions, an interview with a medical doctor and standardized laboratory tests for infections HIV1/2, HBV, HCV, Syphilis (serology and/or nucleic acid testing) and hematological cell counts.

Buffy Coats were produced from whole blood donations on day 1 by using the Top & Bottom Extraction Bag System (Polymed Medical Devices, New Delhi, India). Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats by Biocoll density gradient centrifugation (Biochrom, Berlin, Germany) on day 2. PBMCs were cultured overnight in RPMI 1640 medium (Gibco/Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum gold (PAA Laboratories, Pasching, Austria), 2 mM L-glutamine, 50 units/ml penicillin and 50 µg/ml streptomycin (all Gibco/Life Technologies) at 37 °C in a humid 7.5% CO₂ atmosphere.

Human Leukemic monocyte cell line THP-1 (ATCC® TIB-202) was cultured in RPMI 1640 medium (Gibco/Life Technologies) supplemented with 10% fetal bovine serum (FBS) gold (PAA), 2 mM L-glutamine, 50 units/ml penicillin and 50 µg/ml streptomycin (all Gibco/Life Technologies) at 37 °C in humid 7.5% CO₂ atmosphere.

**Antibodies**

Flow cytometry was performed using the following antibodies from BD Biosciences (Franklin Lakes, NJ, and USA): anti-CD3 (FITC, UCHT1), anti-CD3 (BV605, OKT3), anti-CD8 (PE, RPA-T8) anti-CD69 (PE, L78), anti-CD98 (BV786, UM7F8) and anti-CD161 (APC, DX12). Antibodies against CD8 (Pacific Blue, SK1), CD45RA (PE, HI100), CD56 (PE-Cy5, HCD56), CD107a (PE, H4A3), CCR7 (APC-Cy7, G043H7), GzmA (Alexa Fluor®594, CB9), GzmK (FITC, GM26E7) and Vα7.2 (PE-Cy7, 3C10) were obtained from BioLegend (San Diego, CA, USA).

STED microscopy was performed using unconjugated antibodies obtained from BD Biosciences (anti-CD107a, clone H4A3; anti-Perforin, 6G9), abcam (anti-CD107a, rabbit polyclonal; anti-LAAO, rabbit polyclonal), Santa Cruz (anti-GzmM, clone A-10) and LSBio (anti-GzmB, clone 4E6).

**Fluorescence activated cell sorting**
PBMCs were stained with antibodies against CD3 (FITC), CD8 (PE), CD56 (PE-Cy5), CD161 (APC) and the Vα7.2 chain (PE-Cy7). Cells were then sorted using using a FACSAria II flow cytometer (BD Biosciences; Biozzle size: 70 μm; system pressure: 70 PSI; flow rate 30,000 events/sec; laser: 488 nm with 100 mWatt for FITC, 561 nm with 50 mWatt for PE, PE-Cy5 and PE-Cy7, 640 nm with 60 mWatt for APC; detection with bandpass filters for FITC 525/50, PE 585/15, PE-Cy5 670/14, PE-Cy7 780/60, and APC 670/30). The purity of the sorted cells was assessed by flow cytometry. After sorting, cells were washed with PBS and processed further for mass spectrometric analysis.

Cell lysis, protein digestion and iTRAQ labelling

Sorted cells were lysed in 1 M tetraethylammonium bromide (TEAB) supplemented with 8 M urea for 30 minutes at room temperature (RT), with Benzonase added. After incubation, 5 mM tris(2-carboxyethyl)phosphine (TCEP) was added for 30 minutes, and 10 mM methyl methanethiosulfonate (MMTS) 15 minutes, to reduce and protect cysteins, respectively. Sequence grade, combined LysC/trypsin reagent (Promega, Fitchburg, WI, USA) was added at a ratio of 1:20 weight per weight. After 5 hours incubation at room temperature, the lysate was diluted with MilliQ water from 8 M to 1 M urea and incubated at 37 °C over night. Peptides were then cleaned up via Oasis reverse phase columns (Waters Corporation, Milford, MA, United States). Labeling of tryptic peptides with isobaric iTRAQ reagents was performed according to the manufacturer’s guidelines (Applied Biosystems, Waltham, MA, USA). Samples were vacuum dried and dissolved in 0.2 % trifluoroacetic acid/3 % acetonitrile. Peptides were then cleaned up via Oasis reverse phase columns again, and combined at the same amounts.

Peptide fractionation by Strong Cation Exchange Chromatography (SCX)

The combined iTRAQ-labeled peptide samples were further subfractionated by strong cation exchange chromatography (SCX) to support representative and comprehensive protein identification by LC-MS/MS. Peptides were dissolved in SCX buffer (0.065% formic acid, 25% ACN), fractionated on a Mono SPC1.6/5 column connected to an Ettan micro-LC system (both GE Healthcare, Chicago, IL, USA), and separated at a flow rate of 150 μl/min for 15 min with a linear gradient from 0 % to 35 % SCX buffer supplemented with 0.5 M potassium chloride. Fractions were collected by a microfraction collector every minute (Sunchrom SunCollect, Friedrichsdorf, Germany). Peptide elution was monitored by an UV detector at 214 nm. Peptide-containing fractions were vacuum-dried, cleaned up via Oasis reverse phase columns (Waters Corporation) and analyzed separately by LC-MS/MS.

LC-MS/MS Measurement and Protein Identification

LC-MS/MS analyses of purified and desalted peptides were performed on a Dionex UltiMate 3000 n-RSLC system connected to an Orbitrap FusionTM TribridTM mass spectrometer (Thermo Scientific, Waltham, MA, USA). Peptides of each fraction were loaded onto a C18 pre-column (3 μm RP18 beads, Acclaim, 75 mm x 20 mm), washed for 3 min at a flow rate of 6 mL/min and separated on a C18 analytical column (3-mm, Acclaim PepMap RSLC, 75 mm x 25 cm, Dionex, Sunnyvale, CA, USA) at a flow rate of 350 ml/min via a linear 120 min gradient from 97 % MS buffer A (0.1 % formic acid) to 25 % MS buffer B (0.1 % formic acid, 80 % acetonitrile), followed by a 15 min gradient from 25 % MS buffer B to 62 % MS buffer B. The LC system was operated with the Chromeleon software (version 6.8, Dionex) embedded in the Xcalibur software suite (version 3.0.63, Thermo Scientific). The effluent was electro-sprayed by a stainless steel emitter (Thermo Scientific). Using the Xcalibur
In silico analysis of upstream regulators

Ingenuity Pathway Analyser (Qiagen, Hilden, Germany) was used for determining upstream factors based on the complete proteome data set. As selection criteria all type of immune cells were used and only statistically significant pathways (p <= 0.05) were reported.

Evaluation of proteomic data and statistical analysis

Determination of significantly regulated proteins was performed as described before. [28],[34] In brief, it was assumed that the log2-regulation factors (RF) of each protein follow a normal distribution with different expected values for different proteins, but with the same standard deviation. The mean of the Median Absolute Deviation from the median (MAD) of all proteins serves as a basis for an estimator for the standard deviation. It was then possible to construct a hypothesis test for the identification of significantly regulated proteins in a specific minimum number of donors. The threshold for significance was set as <5 %. Graphs and statistical analyses were completed using R v3.4.1 [65].

Flow cytometric analysis of MAIT cell activation

Target cells (THP-1 or primary monocytes) were fed with PFA-fixed E. coli BL21 over night (MOI 25), and co-incubated with human 500.000 PBMCs for two hours at an E:T ratio of 10:1. Afterwards, cells were washed with 150 µL PBS containing 2 % FBS and 2 mM EDTA, fixed with 100 µL 2 % paraformaldehyde in PBS (20 min, RT) and stained with monoclonal antibodies against CD3, CD107a, CD69, CD161 and Va7.2 (15 min, 4 °C) Analysis of MAIT cell activation was conducted on a BD LSR II SORP cytometer, operated by FACSDiva software (BD Biosciences, v6.1). Data analysis was then carried out by FlowJo (TreeStar, v10.0.7). Gating strategy is shown in Figure 1A.

Intracellular staining of cytokines

For the phenotypic identification, bulk PBMCs were stained with UV Live/Dead Fixable Dead Cell Stain (Invitrogen) and a combination of the following antibodies (from Biolegend except as noted): CD3 (BV605), CD8 (Pacific Blue), CD45RA (PE), CD98 (BV786, BD Biosciences), CD161 (APC, BD Biosciences), CCR7 (APC-Cy7) and the Va7.2 chain (PE-Cy7). Cells were then permeabilized and fixed using BD Cytofix/Cytoperm™ followed by intracellular staining against GzmA (Alexa Fluor®594) and GzmK (FITC). Analysis of MAIT cell activation was conducted on a BD LSR II SORP cytometer, operated by FACSDiva software (BD Biosciences, v6.1). Data analysis was then carried out by FlowJo (TreeStar, v10.4.2) and Prism (GraphPad Software, v7.0c). To determine significant differences, Wilcoxon matched-pairs signed rank test was used. The threshold for significance was set as <5 %.
Confocal and STED Imaging

Two channel images of individual focal planes and 3D image series (stacks) of double-labeled MAIT / THP cells were sequentially recorded using a 3D STED microscope (Leica TCS SP8 STED 3X, Leica Microsystems, Wetzlar, Germany) in either confocal or STED mode, respectively. The microscope was equipped with hybrid photomultipliers (HYD) for sensitive detection, a white light laser (WLL) source for excitation and a pulsed 775 nm laser for emission depletion during STED imaging. To obtain a specific bright and stable fluorescence during STED imaging we used secondary anti-mouse and anti-rabbit antibodies labelled with Abberior Star 580 or Abberior Star 635p (Abberior GmbH, Göttingen, Germany), respectively. Immunocytochemistry was performed due to the manufacturer’s protocol and samples were embedded in Prolong Gold. Regions of interest, i.e. pairs of MAIT/THP cells attaching each other, were scanned unidirectionally by using a 100x oil immersion objective with a numerical aperture of 1.4 (HC PL APO CS2 100x/1.40 OIL). The diameter of the confocal pinhole was set to 141 (= Airy: 0.93) or 151 µm (= Airy: 1) and images were taken at 1024 × 1024 or 2048 × 2048 pixel resolution with zoom factors of 5 or 3, respectively, at a scan rate of 400 Hz with line average of 6. Usually, individual focal planes of each staining were taken sequentially in confocal and STED mode from the center of the cell and overlaid with a DIC image taken by the transmitted light detector. Additionally, 20 to 30 optical planes were scanned in axial direction through the contact zone of the cells in STED mode with a step size of 0.22 µm between two focal planes. These settings resulted in an image volume of 38.7 µm (x) × 38.7 µm (y) × 3.3 µm (z) with a voxel size of 19 nm (x) × 19 nm (y) × 219 nm (z) at 8-bit gray scale resolution.

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Conflict of interest

The authors declare no financial or commercial conflict of interest.

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References


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Figure legends

**Figure 1. MAIT cells from human donor blood are resting and responsive.** (A) Gating strategy for MAIT cells in this study. MAIT cells are defined as CD3⁺Va7.2⁺CD161⁺⁺ lymphocytes. (B&C) Control of MAIT cell responsiveness in PBMCs isolated from human blood. PBMCs were incubated with fixed *E. coli* (MOI 25) for 6h. MAIT cells react to this stimulus with the surface expression of activation markers CD107a (B) and CD69 (C). Data of one representative donor is shown (five separate experiments with one donor each).
Figure 2. Overview over proteomic analysis of primary human MAIT cells. (A) Experimental strategy for this proteomic study. MAIT cells, cCD8\(^+\) T cells and NK cells were sorted from healthy human blood of five independent donors, differentially labeled with iTRAQ reagents, mixed and analyzed by mass spectrometry. (B) In total, 5,600 proteins were identified in one MS experiment, with 2,619 of them being present in all five donors and all three analyzed cell types. (C) Boxplot displaying the distribution of log2 regulation factors (log\(_2\)RF MAIT: cCD8\(^+\)) for all identified proteins. The thick line indicates the median, and boxes the range that contains 50% of the data, while the whiskers indicate the minimum and maximum of the data, as long these do not differ more than 1.5-fold as the interquartile distance from the median. Data points that lie outside this distance are considered as outliers and displayed as points. Box plots show regulatory information comparing MAIT and cCD8\(^+\) T cells of five individual donors. Immune cell subsets of each donor were pooled and analyzed in one proteome experiment per donor (n=5 proteome experiments).
Figure 3. Expression pattern of effector molecules in MAIT cells, cCD8⁺ T cells and NK cells. Distribution of the donor-specific median log₂RF for proteins Granulysin, Granzymes (A, B, H, K, M) and Perforin in five analyzed human donors as determined by proteomics. (A) Regulation factors for MAIT cells vs. cCD8⁺ T cells. (B) Regulation factors for MAIT cells vs. NK cells. Stars indicate significance levels for regulation in all five donors[28]: * p < 0.05, ** p < 0.01, *** p < 0.001 Each dot represents one individual donor in an independent experiment (n = 5, five experiments). (C&D) Granzyme expression in resting T cell subsets. PBMCs were isolated from donor blood and stained with monoclonal antibodies. Granzyme expression was measured by flow cytometry. MAIT: MAIT cells, T_N: naïve CD8 T cells, T_EM: effector memory CD8 T cells, T_EMRA: effector memory CD8 T cells expressing CD45RA, T_CM: central memory CD8 T cells. Horizontal bars indicate mean ± standard deviation. Stars indicate significant differences to the expression in the MAIT cell population determined by Wilcoxon matched-pairs signed rank test: * p < 0.05, ** p < 0.01, *** p < 0.001. Each dot represents the mean of two technical replicates of a healthy donor (C: n = 10, D: n = 8, data are pooled from three independent experiments).
Figure 4. Identification of MAIT cell immunological synapse components. Primary human MAIT cells were coincubated with *E. coli*-fed THP-1 cells for 30 minutes and stained for immunofluorescence analysis. Conjugates were analyzed by STED microscopy. (A-D) Conjugates were labeled with antibodies against granzyme M, granzyme B, CD107a, l-amino-oxidase (LAAO) and perforin, respectively. Representative images from one out of two independent experiments (one donor each) are shown. Scale bar represents 5 µm. DIC: Differential interference contrast.
Mucosal invariant T (MAIT) cells are the highest abundant effector memory T cell subset in humans. With proteomics, we compared MAIT cells to CD8+ T and NK cells in healthy individuals and discovered functions, including granzyme K and LAAO, which contribute to their unique anti-bacterial responsiveness at immunological synapses.
### Table 1. Identified proteins with pronounced expression in MAIT cells

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