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Iron Is Critical for Mucosal-Associated Invariant T Cell Metabolism and Effector Functions

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Mucosal-Associated Invariant T (MAIT) cells are a population of innate T cells that play a critical role in host protection against bacterial and viral pathogens. Upon activation, MAIT cells can rapidly respond via both TCR-dependent and -independent mechanisms, resulting in robust cytokine production. The metabolic and nutritional requirements for optimal MAIT cell effector responses are still emerging. Iron is an important micronutrient and is essential for cellular fitness, in particular cellular metabolism. Iron is also critical for many pathogenic microbes, including those that activate MAIT cells. However, iron has not been investigated with respect to MAIT cell metabolic or functional responses. In this study, we show that human MAIT cells require exogenous iron, transported via CD71 for optimal metabolic activity in MAIT cells, including their production of ATP. We demonstrate that restricting iron availability by either chelating environmental iron or blocking CD71 on MAIT cells results in impaired cytokine production and proliferation. These data collectively highlight the importance of a CD71-iron axis for human MAIT cell metabolism and functionality, an axis that may have implications in conditions where iron availability is limited. ***The Journal of Immunology*****, 2024, 212: 1706–1713.**

Mucosal-Associated Invariant T (MAIT) cells are a population of non–MHC-restricted T cells that are important in the immune defense against bacterial and viral infections (1–5). MAIT cells are rapidly responding T cells that are capable of producing multiple cytokines upon activation, such as IFN-γ, TNF, and IL-17 (1, 6). MAIT cells are activated when their invariant TCR recognizes bacterial riboflavin derivatives presented on the MHC-like molecule MR1 (5, 6). In contrast, they can also be activated in a TCR-independent manner, via stimulation with cytokines such as IL-18 (7, 8). Recently, several studies have highlighted the importance of metabolism for MAIT cell functional responses (9). Our group and others have demonstrated that MAIT cells are reliant on glycolysis to support their production of IFN-γ and granzyme B (10–12), whereas IL-17 production by MAIT cells has been linked to mitochondrial metabolism (13, 14). However, our knowledge of the nutritional requirements of MAIT cells remains limited.

Iron is an essential trace element for all multicellular organisms and is critical for a range of physiological processes, including oxygen transport and energy production (15). Iron is also critical for the majority of microorganisms, and successful iron sequestration is required to establish infection (16). Iron availability is regulated by the liver-derived hormone hepcidin. Increased hepcidin production occurs in response to infection and inflammation, and results in reduced iron availability, a mechanism of host protection (17, 18). However, previous studies have demonstrated that conventional T cell responses are negatively impacted by low iron levels, with reduced proliferation and effector functions (19, 20). Whether MAIT cells require iron for their metabolic processes and effector responses is currently unknown.

In this study, we demonstrate that MAIT cells upregulate their expression of the transferrin receptor, CD71, upon activation, correlating with a significant increase in the uptake of transferrin-bound iron. In silico analysis of the MAIT cell proteome reveals that MAIT cells increase their overall iron content upon activation. We demonstrate that MAIT cell metabolism is limited, and the metabolic profile altered, under iron-deplete conditions, and this is associated with a robust reduction in ATP levels. Finally, we

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Abbreviations used in this article: DFO, deferoxamine; FCCP, carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone; HPLM, human plasma like media; MAIT, Mucosal-Associated Invariant T; MFI, mean fluorescence intensity.

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demonstrate that extracellular iron restriction impairs MAIT cell functional responses, lending to a reduced proliferative capacity and diminished cytokine production. Collectively, our data pinpoint MAIT cells as another player in the iron tug-of-war between pathogenic invaders and host immunity.

Materials and Methods

Study cohorts and ethical approval

A total cohort of 30 healthy adult donors was recruited. Inclusion criteria included ability to give informed consent, 18–65 y of age, body mass index- < 28, and no current or recent (<2 wk) infection. Ethical approval was obtained from both St Vincent's University Medical Ethics Committee and Maynooth University Ethics Committee.

Preparation of PBMCs and flow cytometric analysis

PBMC samples were isolated, using SepMate isolation tubes, by density centrifugation over Lymphoprep (both STEMCELL Technologies), from fresh human peripheral blood samples. Cell viability was determined using eBioscience Fixable Viability Dye (eFluor506), and MAIT cells were phenotyped using specific extracellular mAbs (Miltenyi Biotec and BioLegend), namely, CD3, CD161, TCRVα7.2, and CD71. Where appropriate, cells were fixed and permeabilized according to manufacturer guidelines, using the True-Nuclear Transcription Factor Buffer set (BioLegend). Cell populations were identified using an Attune NXT flow cytometer and analyzed using FlowJo version 10.8.2 (TreeStar). Results are expressed either as a percentage of the parent population as indicated and determined using flow minus one and unstained controls, or as the mean fluorescence intensity (MFI) of the relevant population.

MAIT cell transferrin uptake assay

PBMCs (2×10^6/ml) were activated using CD3/CD28 TCR Dynabeads (Life Technologies) and IL-18 (50 ng/ml) or cytokine alone (IL-12/IL-18, both 50 ng/ml) for 18 h as indicated. Cells were rested in serum-free human plasma like media (HPLM) with 5% BSA for 2 h. Cells were then washed in serum-free HPLM with 0.5% BSA and incubated with 5 μg/ml Transferrin-AlexaFluor647 (Invitrogen) for 10 min at 37°C. Holo-transferrin (500 μg/ml; Sigma-Aldrich) was used to competitively control for transferrin uptake. Cells were washed in ice-cold HPLM with 0.5% BSA to stop membrane trafficking. Cells were then stained for viability, and MAIT cells or conventional T cells were labeled for extracellular markers, to be analyzed by flow cytometry.

In silico proteomic analysis

A publicly available proteomic dataset of MAIT cells was downloaded from PRIDE accession number PXD041544 (https://www.ebi.ac.uk/pride/archive/projects/PXD041544). A list of human iron-interacting proteins was provided by Andreini et al. (21). Using the protocol from Teh et al. (22), the list of human iron-interacting proteins was compared and aligned against the complete list of proteins detected in the human MAIT cell proteomic dataset. Matches were extracted and listed in Supplemental Table I. To estimate the iron atom counts per protein species, we multiplied the copy number value of each iron-interacting protein by the iron atom counts per protein. If available, iron atom counts per protein were obtained using the UniProt database cofactor information for each protein. Where iron counts were not available, estimates of iron usage per protein species were assumed to be 1 atom for heme and iron-interacting proteins and 2 atoms for FE-S cluster-interacting proteins. The total number of iron atoms required per cell was calculated as the sum of iron atoms required by each protein species.

MAIT cell SCENITH assay

Fresh PBMCs (2×10^6/ml) were activated using CD3/CD28 TCR Dynabeads and IL-18 (50 ng/ml) for 18 h. Cells were seeded into a 96-well plate, and treated as a control, or with 2-deoxy-D-glucose (100 mM), oligomycin (1 μM), or both. After incubation at 37°C for 15 min, cells were treated with puromycin (11 μM) and incubated for a further 25 min. Cells were washed with ice-cold PBS to stop puromycin incorporation. Cells were then stained for viability. MAIT cells were stained for extracellular markers and fixed, as outlined earlier. Staining of puromycin was achieved using anti-puromycin mAb (AlexaFluor488; Sigma) in permeabilization buffer (BioLegend).

MAIT cell Seahorse assay

Purified MAIT cells (IL-2 expanded) were activated using CD3/CD28 TCR Dynabeads and IL-18 (50 ng/ml) for 18 h in the absence or presence of deferoxamine (DFO; 200 μM; Sigma), and metabolic analysis was carried out using the Seahorse Extracellular Flux Analyzer XFe96 (Agilent). MAIT cells were resuspended in phenol red–free RPMI media containing 10 mM glucose, 2 mM glutamine, and 1 mM pyruvate (Agilent) and plated onto a Cell-Tak (Corning)-coated microplate for adhesion. Respiratory parameters (mitochondrial and glycolytic) were measured using oxygen consumption rate (pmol/min) and extracellular acidification rate (mpH/min), respectively, using injections of oligomycin (1 μM), carbonyl cyanide-4 (trifluoromethoxy) phenylhydrazone (FCCP) (1 μM), rotenone and antimycin A (both 1 μM), and monensin (20 μM) (all Sigma). Metabolic parameters were calculated as per well-established protocols (23, 24).

MAIT cell mitochondrial analysis

Fresh PBMCs (2×10^6/mL) were activated using CD3/CD28 TCR Dynabeads and IL-18 (50 ng/ml) for 18 h in the absence or presence of DFO (200 μM; Sigma). Cells were seeded into a 96-well plate and washed in serum-free buffer. Cells were then stained for viability as outlined earlier, and MAIT cells were stained for extracellular markers. Cells were then washed and stained with MitoTracker Deep Red FM (50 μM; ThermoFisher Scientific) and MitoTracker Green (50 μM; ThermoFisher Scientific) in PBS and incubated for 1 h at 37°C. Cells were subsequently analyzed by flow cytometric analysis.

MAIT cell ATP assay

IL-2 expanded MAIT cells (1×10^6/ml) were stimulated using CD3/CD28 TCR Dynabeads and IL-18 (50 ng/ml) for 18 h. ATP levels were measured using a luminescence ATP assay kit (Abcam). Reagents in the kit were reconstituted as per manufacturer's instructions. A standard curve was also prepared as per the kit's instructions. MAIT cells were harvested and washed with PBS. A total of 100 μl of resuspended MAIT cells was added to a black-walled, clear-bottomed plate. A total of 50 μl of detergent was added to each well, and the plate was placed on an orbital shaker for 5 min at 600–700 rpm. A total of 50 μl of substrate solution was added, and the plate was returned to the orbital shaker for 5 min at 600–700 rpm. The plate was then covered and placed in the dark for 10 min before luminescence was measured on a multimode plate reader (CLARIOstar).

MAIT cell functional analysis

IL-2 expanded MAIT cells were activated using CD3/CD28 TCR Dynabeads and IL-18 (50 ng/ml) for 18 h in the absence or presence of DFO (100 μM) or anti-CD71 mAb (20 μg/ml). An appropriate IgG isotype control was used in blocking experiments. After 18 h, culture supernatants were assessed for IFN-γ, IL-17, or IL-26 levels using ELISA.

MAIT cell Escherichia coli *stimulation assay*

Freshly isolated MAIT cells were incubated with 20 μg/ml anti-CD71 mAb (Invitrogen) for 1 h. Meanwhile, THP-1 cells (ATCC TIB-202) were prepulsed for 1 h with fixed *E. coli* (DH5α) at a multiplicity of infection of 100. At the end of an hour, representative THP-1 cells with and without *E. coli* were counted and titrated to match the number of MAIT cells at a 1:1 ratio, and MAIT cells were cocultured with THP-1 overnight. Four hours before staining for flow cytometry, cells were centrifuged and resuspended in media containing brefeldin A (Invitrogen). Intracellular cytokine (IFN-γ and granzyme B) levels were assessed using flow cytometry (Cytex Aurora).

MAIT cell proliferation analysis

Fresh PBMCs (1×10^6/ml) were stimulated for 24 h with 5 μg/ml 5-Amino-6-(D-ribitylamino)uracil and 100 μM methylglyoxal, in the absence or presence of DFO (200 μM) and $FeSO_4 \bullet 7H_2O$ (200 μM; Sigma). After 24 h, culture media were replaced with fresh culture media containing IL-2 (6.8 ng/ml). After 48 h, culture media were replaced with fresh culture media containing IL-2 (34 ng/ml). On day 5, absolute cell numbers were determined using flow cytometric analysis of MAIT cell frequencies and total cell counts.

Statistics

Statistical analysis was completed using GraphPad Prism 6 Software. Data are expressed as SEM. Distribution was assessed using Shapiro–Wilk test. We determined differences between two groups using Student *t* test (paired or unpaired) or Wilcoxon signed-rank test where appropriate. Analysis across three or more groups was performed using ANOVA with multiple measures. Correlations were determined using linear regression models and expressed using Pearson or Spearman rank correlation coefficient, as appropriate. The *p* values were expressed with significance set at <0.05.

Results

MAIT cells increase their expression of the transferrin transporter CD71 upon activation

First, we demonstrate that MAIT cells from PBMCs increase transferrin receptor (CD71) surface expression in response to TCR/IL-18 stimulation, but not cytokine alone (Fig. 1A–D, Supplemental Fig. 1). We then compared CD71 expression on activated MAIT cells and activated conventional T cells and noted higher CD71 expression on conventional T cells (Fig. 1E). Next, we investigated whether MAIT cells altered their CD71 expression under restricted iron conditions. To do this, we limited iron availability in our culture system using the iron chelator DFO, which did not impact MAIT cell viability at 18 h (Supplemental Fig. 2). Resting MAIT cells do not have high levels of CD71 expression; however, upon iron depletion, both the proportion of MAIT cells that express CD71 and the amount of CD71 expressed were increased, and this was also evident in TCR/IL-18 activated MAIT cells (Fig. 1F–H, Supplemental Fig. 2), suggesting a compensatory mechanism to support iron uptake. Next, we investigated the functionality of CD71, using a transferrin uptake assay; showed increased transferrin uptake (both % of MAIT cells and MFI) by activated MAIT cells; and again noted higher uptake by activated conventional T cells (Fig. 1I–M). Finally, we demonstrated a linear relationship between CD71 expression and transferrin uptake in activated MAIT cells (Fig. 1N).

In silico analysis of the MAIT cell proteome reveals increased iron content upon activation

To assess the iron requirements of MAIT cells, we performed in silico pathway analysis of a previously published proteomics dataset (12). We found that IL-2 expanded MAIT cells express high basal expression of ferritin, the protein responsible for iron storage, which is reduced upon activation (Fig. 2A, 2B). Next, we determined the number of iron-interacting proteins in MAIT cells and identified 135 proteins in total, representative of 2.3% of the total proteome (Fig. 2C, Supplemental Table I). Pathway analysis revealed these proteins were intrinsic to many processes, including a major involvement in cellular metabolism (Fig. 2D). Using a recently published algorithm (22) that determines cellular iron content, based on number of iron-containing proteins multiplied by the number of iron atoms per protein, we determined the predicted iron content from the MAIT cell dataset and showed $\sim 1 \times 10^7$ iron atoms in MAIT cells, increasing significantly upon activation (Fig. 2E). Finally, we found that the iron content of MAIT cells is associated with heme and iron-sulfur clusters (Fig. 2F, 2G).

Iron is a critical cofactor for MAIT cell metabolism

We next investigated whether limiting iron availability in our culture system using the iron chelator DFO impacted MAIT cell metabolism. Using protein translation as functional readout of metabolism, we demonstrate that activated MAIT cells significantly increase protein translation after stimulation (Fig. 3A, 3B), and protein translation is limited when MAIT cells are activated in the presence of DFO (Fig. 3C, 3D, Supplemental Fig. 2). A recently published method, SCENITH (25), demonstrated that the measurement of protein translation, paired with the use of metabolic inhibitors such as 2-deoxyglucose (2DG) and oligomycin, allows for metabolic analysis of cells at a single-cell level. Using the SCENITH approach (Supplemental Fig. 2), we demonstrate that iron-restricted MAIT cells increase their dependency on glucose metabolism (Fig. 3E), and this was paired with a decreased capacity to undergo oxidative-linked metabolic processes (Fig. 3F). With this reduction in oxidative metabolism, we next examined the impact of limiting iron on the mitochondrial phenotype, and we observed modest increases in both mitochondrial mass (MitoTracker Green) and membrane potential (MitoTracker Deep Red) (Fig. 3G, 3H). To confirm these observations, we next investigated the impact of iron restriction via DFO treatment on MAIT cell metabolism using extracellular flux analysis, and we noted strong inhibition in the rates of oxidative phosphorylation (Fig. 3I, 3J). We also noted reduced rates of glycolysis (Supplemental Fig. 3). Furthermore, when we tested the mitochondrial capacity of MAIT cells using the FCCP treatment, we observed a striking reduction with DFO treatment (Fig. 3K). Finally, we examined the impact of low iron availability, using an mAb to block CD71, on ATP levels in MAIT cells, and we demonstrated that iron restriction limits ATP production (Fig. 3L), a finding supported by our Seahorse analysis (Supplemental Fig. 3).

MAIT cell functional responses require extracellular iron

We next investigated whether MAIT cells require extracellular iron for their functional responses. We assessed the impact of DFO treatment on MAIT cell in PBMC ability to produce IFN-γ via flow cytometry, and we observed a reduction with iron restriction (Fig. 4A). Next, we investigated the impact of DFO on the cytokine responses of isolated MAIT cells and show reductions in IFN-γ, IL-17, and IL-2 levels (Fig. 4B–D). To confirm the impact of low iron availability on MAIT cell IFN-γ production, we switched our approach to block CD71 and again demonstrate significant reductions in IFN-γ levels (Fig. 4E). We next assessed the impact of limiting iron availability on MAIT cell production of the antimicrobial cytokine IL-26, and we demonstrated significantly reduced levels with CD71 blockade (Fig. 4F). We next assessed the impact of iron restriction via CD71 blockade on freshly isolated MAIT cell responses to THP-1 cells infected with *E. coli*, and we demonstrated significant reductions in both IFN-γ and granzyme B production (Fig. 4G, 4H). With the noted impact of iron restriction on MAIT cell metabolism, we sought to assess the impact of long-term iron restriction on MAIT cell proliferative capacity and viability. We first showed that MAIT cell proliferation is inhibited with the addition of the ATP synthase inhibitor oligomycin (Fig. 4I) and then demonstrated that MAIT cells fail to proliferate under low iron conditions, and this was paired with a significant reduction in cell viability, which was significantly improved with the addition of $FeSO_4$, which bypasses CD71 (Fig. 4J, 4K).

Discussion

MAIT cells are a subset of unconventional T cells capable of rapidly responding to stimulation, producing cytokines and lytic molecules, and proliferating (26). MAIT cells are key mediators of host protection against many bacterial and viral pathogens (2, 3, 27–29). Immune responses are metabolically intense processes, with significant energy demands required to support de novo generation of biosynthetic intermediates (30). The metabolic processes and nutrient requirements that govern MAIT cell effector responses are rapidly emerging but still incomplete (10–13, 31). Iron is an essential microelement and is critical for almost all living organisms, including humans and the majority of microbes (15). Iron plays a critical role in cellular metabolism and energy production, and it is vital for conventional T cell immunity (19, 20). The iron requirements of MAIT cells are unknown. In this study, we show that MAIT cell metabolism and functional responses, namely, cytokine production and proliferation, are governed by extracellular iron availability. We show that activated MAIT cells increase their expression of CD71, the transferrin receptor, and that iron restriction significantly diminishes MAIT cell protein translation and ATP production. Consequently, MAIT cells present with a reduced capacity to proliferate

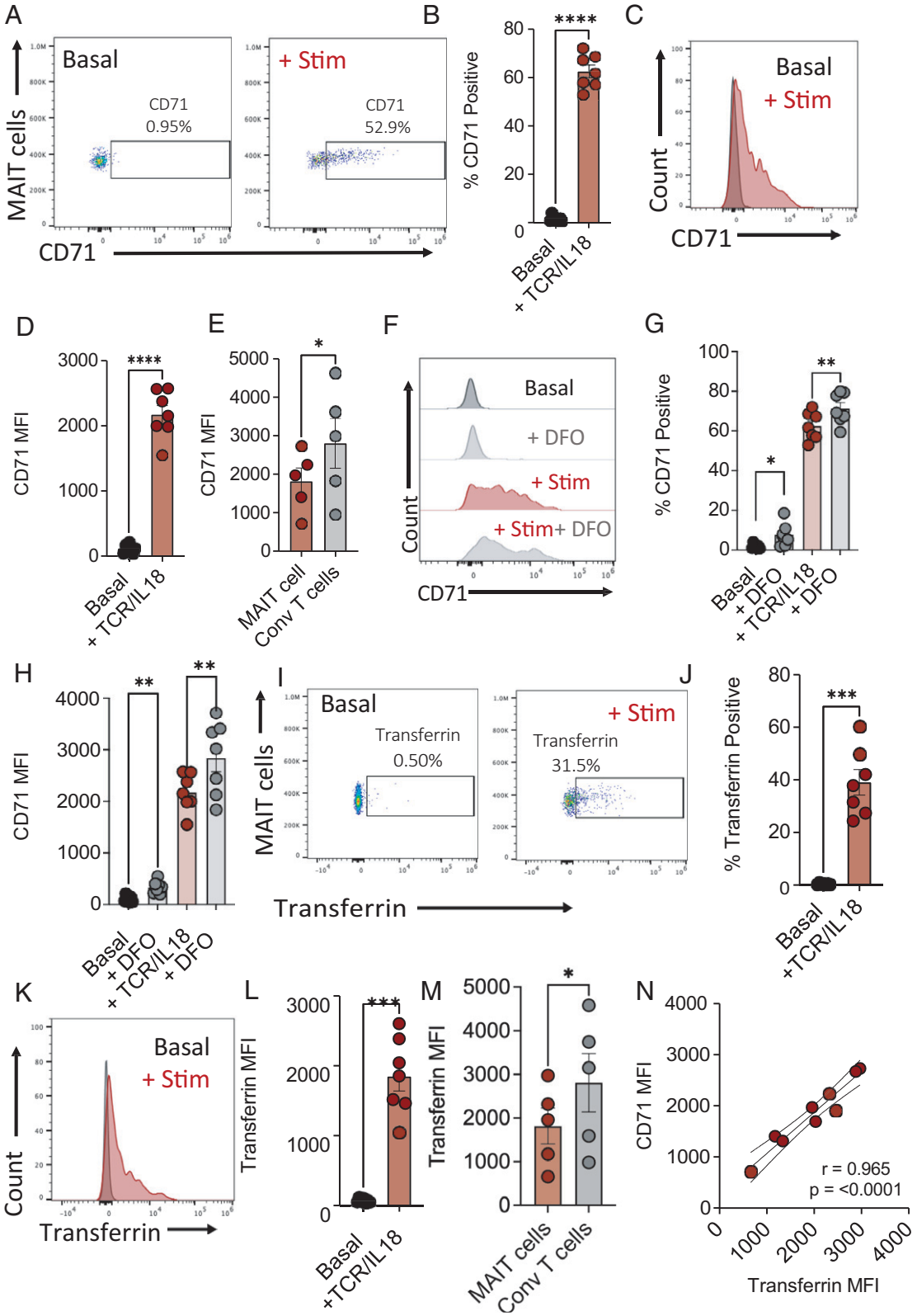

FIGURE 1. MAIT cells increase CD71 and transferrin uptake upon activation. (**A**–**D**) Flow cytometric dot plots, scatterplots, and representative flow cytometric histograms showing CD71 expression in MAIT cells, basal or stimulated for 18 h with anti-CD3/CD28 TCR beads and IL-18. (**E**) Scatterplot comparing CD71 expression on MAIT cells and conventional T cells (non-MAIT $CD3^+$ cells) after 18-h stimulation with anti-CD3/CD28 TCR beads and IL-18. (**F**–**H**) Flow cytometric dot plots, scatterplots, and representative flow cytometric histograms showing CD71 expression in MAIT cells, basal or stimulated for 18 h with anti-CD3/CD28 TCR beads and IL-18, in the absence or presence of the iron chelator, DFO. (**I**–**L**) Flow cytometric dot plots, scatterplots, and representative flow cytometric histograms showing transferrin content in MAIT cells, basal or stimulated for 18 h with anti-CD3/CD28 TCR beads and IL-18. (**M**) Scatterplot comparing transferrin uptake by MAIT cells and conventional T cells (non-MAIT $CD3^+$ cells) after 18-h stimulation with anti-CD3/CD28 TCR beads and IL-18. (**N**) Correlation plot showing the relationship between CD71 expression and transferrin uptake in activated MAIT cells (18 h with anti-CD3/CD28 TCR beads and IL-18). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

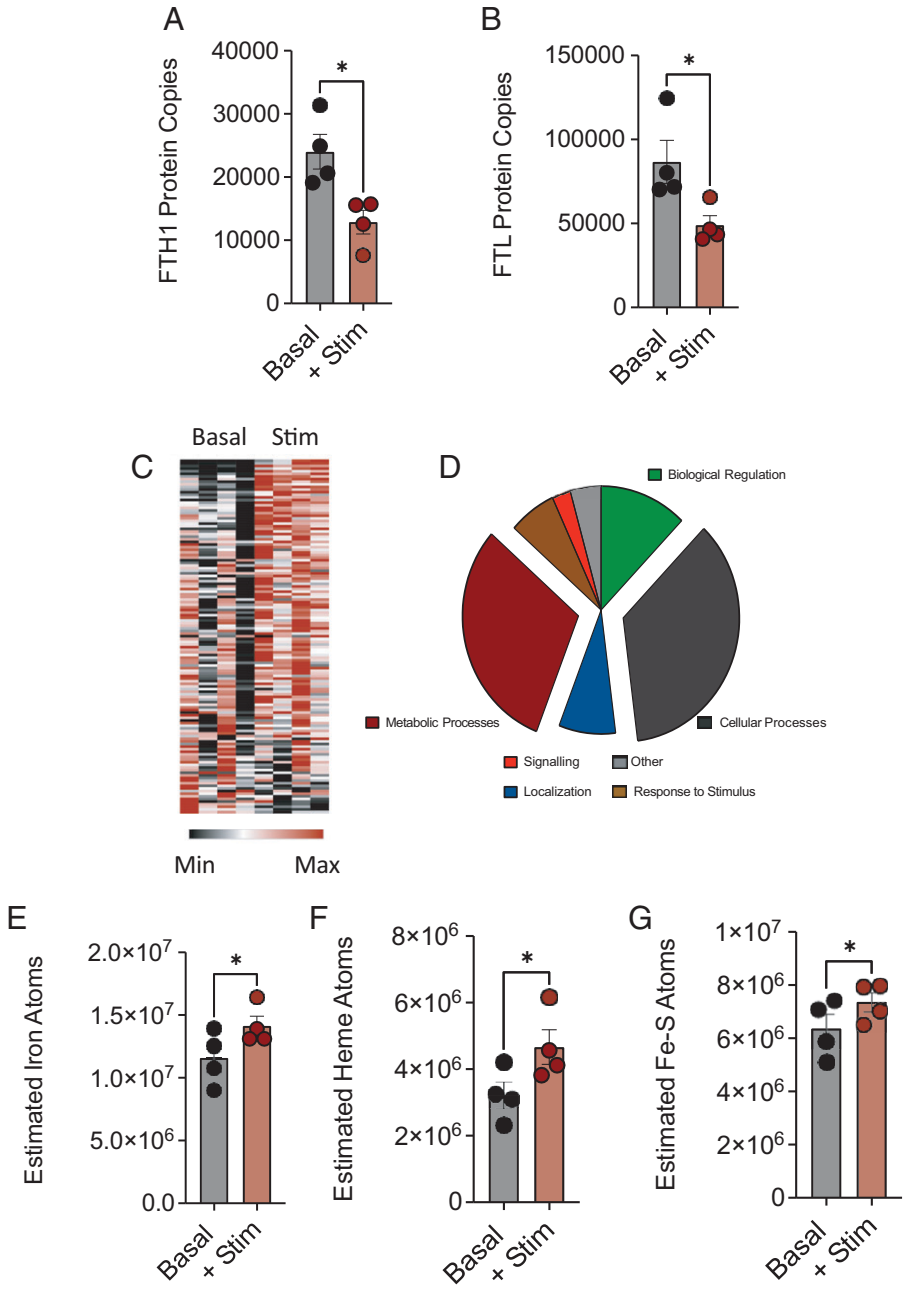

FIGURE 2. MAIT cells store and use iron. (**A** and **B**) Scatterplot showing protein copy number of the H and L chain subunits of ferritin in IL-2 expanded MAIT cells, basal or stimulated for 18 h with anti-CD3/CD28 TCR beads and IL-18. (**C**) Heatmap of iron-related protein content in IL-2 expanded MAIT cells, basal or stimulated for 18 h with anti-CD3/CD28 TCR beads and IL-18. (**D**) Pie graph showing proportional pathway analysis based on iron-related protein content in MAIT cells (analysis performed with Panther). (**E**–**G**) Estimated iron, heme, and iron-sulfur cluster atoms extrapolated from proteome of IL-2 expanded MAIT cells, basal or stimulated for 18 h with anti-CD3/CD28 TCR beads and IL-18. $*p < 0.05$.

and diminished production of several cytokines central to MAIT cell host protective function.

The majority of bioavailable iron in the circulation is bound to transferrin, which is taken into cells via the transferrin receptor TfR1, also known as CD71 (32). We first examined MAIT cells for the expression of CD71 and found low basal expression but significant increases in expression after stimulation with TCR beads/IL-18, but not IL-12/IL-18, suggesting a TCR dependency; this supports our previous study that highlighted CD71 as an MYC target, which is an important transcription factor upregulated by TCR stimulation in MAIT cells (12). Previous studies have reported similar activation-induced increases in CD71 expression on activated T cells and NK cells (33, 34). Interestingly, a missense mutation in *TFRC*, the gene encoding CD71, resulted in immunodeficiency underpinned by defective T cells (35). In line with increased CD71 expression, we observed increased uptake of transferrin by activated MAIT cells, similar to that reported in both conventional $CD4^+$ and $CD8^+$ T cells (36). MAIT cells are primarily $CD8^+$, and recently it was demonstrated that CD8 receptor expression was critical for optimal TCR-driven responses in MAIT cells (37). In a recent study by Teh and colleagues (22), they reported greater iron dynamics in $CD8^+$ T cells compared with $CD4^+$ T cells, which is supported by proteomic data from Howden et al. (33), where they demonstrated that $CD8^+$ T cells express more CD71 than $CD4^+$ T cells. To investigate the iron dynamics of MAIT cells, we performed in silico analysis of our publicly available proteomic dataset (12). We found that MAIT cells decrease their levels of ferritin, suggesting mobilization of iron stores (38). Using the approach introduced by Teh et al. (22) for determining cellular iron content based on proteomic analysis, we found that MAIT cells contain 135 proteins with iron binding sites and significantly increase their predicted iron content upon activation, similar to that observed in conventional T cell subsets (22).

Pathway analysis of the 135 proteins with iron binding sites present in MAIT cells highlighted cellular metabolism as a major process central to these proteins. Similarly, Teh et al. (22) found that the majority of intracellular iron in T cells was used in oxidative

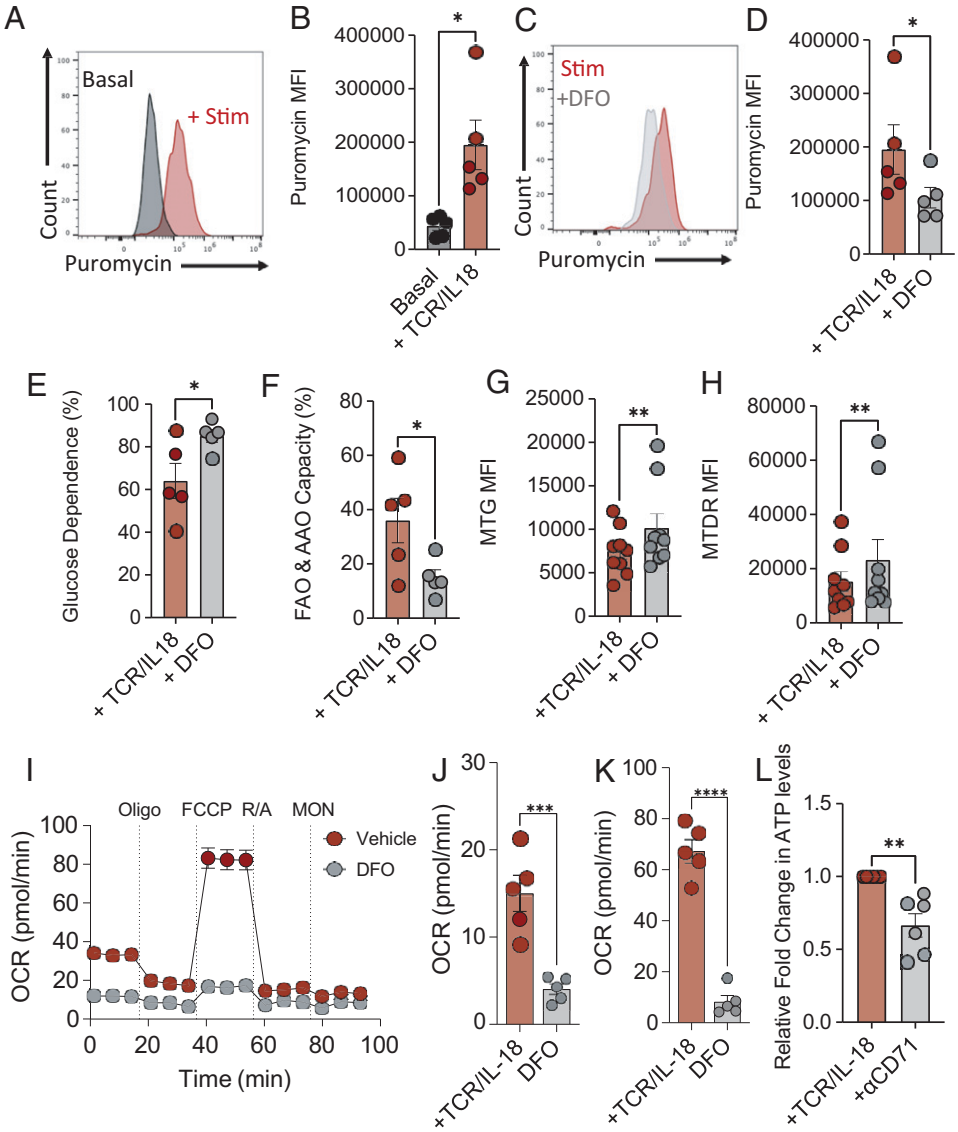

FIGURE 3. Iron restriction alters MAIT cell metabolism. (**A**–**D**) Representative flow cytometric histograms and scatterplots showing puromycin incorporation in MAIT cells, basal or stimulated for 18 h with anti-CD3/CD28 TCR beads and IL-18, in the absence or presence of the iron chelator, DFO. (**E**) Scatterplot showing percentage dependency on glucose metabolism in MAIT cells stimulated for 18 h with anti-CD3/CD28 TCR beads and IL-18, in the presence or absence of DFO. (**F**) Scatterplot showing percentage dependency on fatty acid oxidation and amino acid oxidation in MAIT cells stimulated for 18 h with anti-CD3/CD28 TCR beads and IL-18, in the presence or absence of DFO. (**G** and **H**) Scatterplots showing the MFI of MitoTracker Green and MitoTracker Deep Red, depicting mitochondrial mass and mitochondrial membrane potential, respectively, in MAIT cells stimulated with anti-CD3/CD28 TCR beads and IL-18 for 18 h, in the presence or absence of DFO. (**I** and **J**) Representative Seahorse trace and scatterplot showing oxygen consumption rates (OCRs) in MAIT cells stimulated with anti-CD3/CD28 TCR beads and IL-18 for 24 h, in the presence or absence of DFO. (**K**) Scatterplot showing the mitochondrial capacity (after FCCP treatment) in MAIT cells stimulated with anti-CD3/CD28 TCR beads and IL-18 for 24 h, in the presence or absence of DFO. (**L**) Scatterplot showing the impact of an anti-CD71 mAb with blocking activity on ATP production in MAIT cells stimulated with anti-CD3/CD28 TCR beads and IL-18 for 18 h. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

phosphorylation. Therefore, we next assessed the impact of altered iron availability on MAIT cell metabolism and functional responses. We used two different approaches to limit MAIT cell access to extracellular iron: (1) we used the iron chelator DFO, which removes iron from ferritin (39); and (2) we used an mAb specific for CD71 with blocking activity (40). To assess the impact of low iron availability on MAIT cell metabolism, we used two different approaches: Seahorse extracellular flux analysis and a recently published method, SCENITH, which monitors rapid changes in protein translation paired with a series of metabolic inhibitors to profile cellular metabolism at a single-cell level (25). Using SCENITH, we found that MAIT cells significantly increase protein translation upon activation. This supports our recent publication where we demonstrated significant increases in protein content in activated MAIT cells (12). In the presence of DFO, protein translation was reduced in activated MAIT cells, suggesting that iron supports global MAIT cell metabolism. This was confirmed by our Seahorse data, which showed reduced rates of glycolysis and to a greater extent reduced rates of Oxidative Phosphorylation. Similarly, in activated NK cells, treatment with DFO resulted in reduced cell size, indicative of reduced protein content and metabolism (34). Paired with reduced protein translation, limited iron availability also reduced the oxidative capacity of activated MAIT cells, suggesting altered mitochondria, similar to that reported in conventional T cells by Frost et al. (20). In the same study by Frost et al. (20), iron deficiency resulted in reduced ATP production and an

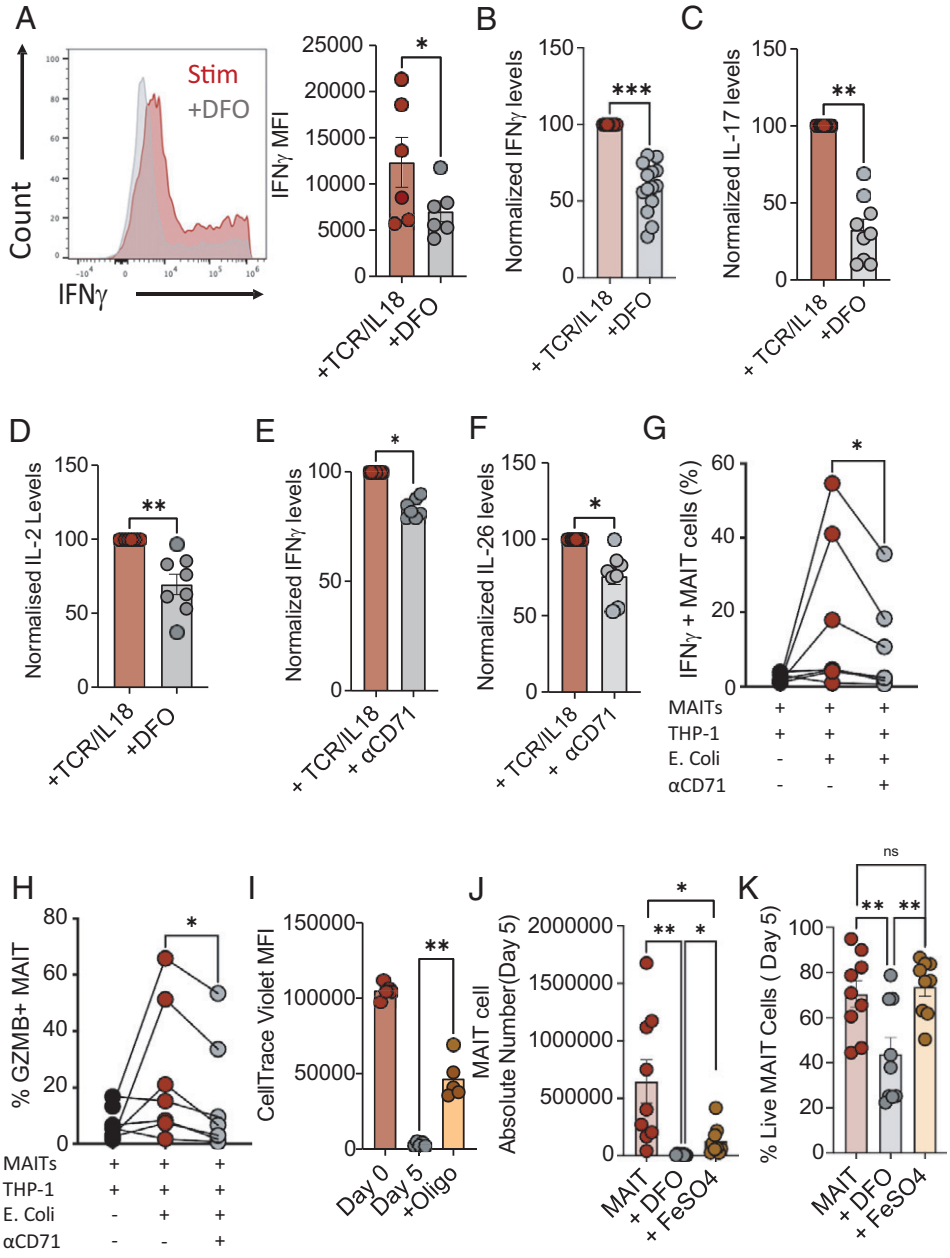

FIGURE 4. MAIT cells require iron for their functional responses. (**A**) Representative flow cytometry histogram and scatterplot showing IFN-γ levels (MFI) in MAIT cells stimulated with anti-CD3/CD28 TCR beads and IL-18 for 18 h, in the absence or presence of the iron chelator, DFO, measured by flow cytometry. (**B**–**D**) Scatterplots showing IFN-γ, IL-17, and IL-2 secreted protein levels (normalized to assay) in IL-2 expanded MAIT cells stimulated with anti-CD3/CD28 TCR beads and IL-18 for 18 h, in the absence or presence of DFO, as measured by ELISA. (**E** and **F**) Scatterplots showing the impact of an anti-CD71 mAb with blocking activity on IFN-γ and IL-26 secreted protein levels (normalized to assay) in IL-2 expanded MAIT cells stimulated with anti-CD3/CD28 TCR beads and IL-18 for 18 h, as measured by ELISA. (**G** and **H**) Line graph showing IFN-γ or granzyme B levels (percentage of parent population) in MAIT cells cocultured at 1:1 ratio with THP-1 cells alone or prepulsed with fixed *E. coli* (DH5α) for 18 h, in the absence or presence of the anti-CD71 mAb with blocking activity, as measured by flow cytometry. (**I**) Scatterplot showing the impact of the ATP synthase inhibitor, oligomycin, on MAIT cell proliferative capacity (during a 5-d IL-2–mediated MAIT cell expansion culture), whereby the MFI of the intracellular dye CellTrace Violet decreases with each cycle of cell division. (**J** and **K**) Scatterplots showing the impact of long-term iron depletion via DFO and alternative iron repletion via Iron(II) sulfate heptahydrate ($FeSO_4 \bullet 7H_2O$) on MAIT cell proliferation and viability during a 5-d IL-2–mediated MAIT cell expansion culture. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

accumulation of dysregulated mitochondria in $CD8^+$ T cells. We observed only modest changes in mitochondrial phenotype, but note the longer time frame in the study by Frost et al. (20) compared with our overnight time point. We did, however, find reduced mitochondrial capacity and ATP levels in MAIT cells activated in the presence of limited iron availability.

Iron is critical for the majority of commensal and pathogenic bacteria, and during infection, in response to inflammation, the host restricts the accessibility of iron as a protective measure. Many pathogenic bacteria synthesize iron chelators called siderophores to scavenge host iron, resulting in an iron tug-of-war (41). One of the major roles for MAIT cells is mediating host protection against bacteria (27, 42–44). Interestingly, several bacteria that elicit MAIT cell responses in vivo, such as *Salmonella typhimurium* (45), *Klebsiella pneumoniae* (46), and *Legionella longbeachae* (47), use siderophores as a virulence factor (48–50). Therefore, we investigated whether MAIT cells need iron for their effector functions, the primary of which is cytokine production. Previously, we had found IFN-γ to be the most abundant cytokine produced by MAIT cells, and in this study we demonstrate that limiting iron availability limits the levels of IFN-γ secreted in response to either TCR-stimulating beads or THP-1 cells infected with *E. coli*. Similarly, in a model of hepcidin-driven hypoferremia, Ag-specific CD8 T cells produced less IFN-γ than in control mice, highlighting the importance of iron for conventional T cell responses. IL-26 is a cytokine with direct antimicrobial activity produced by MAIT cells and part of MAIT cell responses to infection in the lung (28). We found that IL-26 production by MAIT cells was also reduced under limited iron conditions. Another key effector function required for MAIT cell host defense is the ability to proliferate (51). We found that under iron restriction, MAIT cells failed to proliferate, likely linking into the reduced metabolism observed. In CD4 T cells, iron was also required for proliferation, and the authors linked this failure to altered mitochondrial metabolism (19), supporting our data, which demonstrated significant reduction in MAIT cell proliferation with ATP synthase inhibition. In summary, our study highlights the importance of iron for MAIT cell metabolic and functional responses and may have implications in conditions where iron availability is limited.

Disclosures

The authors have no financial conflicts of interest.

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