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IFN γ regulates MR1 transcription and antigen presentation

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Introduction: Antigen presentation molecules play key roles in T cell immunity. Multiple complementary pathways are known to regulate classical MHC-I molecules at transcriptional, translational, and post-translational levels. Intracellular trafficking mechanisms dictating post-transcriptional regulation of MR1, the MHC-I-like molecule which restricts MAIT cells, have been an area of focus; however, little is known about MR1 transcriptional regulation. We demonstrate that interferons regulate MR1 transcription.

Methods: Primary human airway epithelial cells (AEC) were treated with recombinant interferons or co-cultured with MAIT cell clones and antigen sources. MR1 expression was analyzed by RT-qPCR and flow cytometry. MAIT cell activity was quantified by ELISPOT.

Results: Treatment of AECs with IFN β or IFN γ variably increased MR1 transcripts, while only IFN γ significantly increased surface MR1 expression and enhanced antigen presentation to MAIT cells. The MR1 promoter contains binding motifs for interferon regulatory factor 1 (IRF1), an important MHC-I transcription factor. IRF1 knockout reduced IFN γ -stimulated MR1 transcription, surface expression, and antigen presentation. Conversely, knockout of Nod-like Receptor family CARD domain-containing 5 (NLRC5), a critical component of MHC-I transcription, did not significantly impact MR1 expression. These findings were corroborated with IFN γ -treated primary AEC. MAIT cells in co-culture with *Streptococcus pneumoniae*-infected AEC produced sufficient IFN γ to stimulate MR1 expression.

Conclusion: Our data support a model where IFN γ from activated MAIT cells or another source stimulates IRF1-dependent MR1 expression and antigen presentation, leading to greater MAIT cell activation. A robust MR1-dependent MAIT cell response may be beneficial for early infection responses, allowing minimal antigen stimulus to generate greater proinflammatory activity.

KEYWORDS

MAIT (mucosal-associated invariant T) cell, MR1, airway epithelial cell, interferon-gamma, IRF1

Introduction

Mucosal-associated invariant T (MAIT) cells, an innate-like subset of T lymphocytes that comprise a relatively large proportion of the total CD8⁺ T cell population in human blood and lungs, play key roles in clearing respiratory bacterial, fungal, and viral infections (1–3). Upon antigen presentation, MAIT cells are capable of immediate effector function and release inflammatory cytokines like interferon- γ (IFN γ) and tumor necrosis factor (TNF α) (2–5). This rapid activation primes MAIT cells to coordinate early infection response, but also necessitates tight regulation of antigen presentation to prevent inappropriate MAIT cell activation to inappropriate stimuli.

MAIT cells are restricted by the MHC class I-related molecule MR1, which presents small molecule metabolite antigens such as those generated during bacterial riboflavin biosynthesis (2, 3, 6, 7). There is a large pool of potential MR1 ligands produced by commensal airway flora in addition to pathogenic respiratory microbes. *MR1* mRNA is expressed across cell types and tissues, and MR1 proteins primarily reside in intracellular compartments like the ER and endosomal compartments (7–11). The basal intracellular location of MR1 and ligand-induced translocation to the cell surface play critical parts in regulation of MAIT activation (as reviewed in (12–14)).

The intracellular trafficking mechanisms dictating post-transcriptional regulation of MR1 have been an area of research focus; however, little is known about *MR1* transcriptional regulation. Multiple complementary pathways regulate classical MHC-Ia molecules at transcriptional, translational, and post-translational levels (15, 16). Interferons (IFNs) like IFN β and IFN γ drive transcription of MHC-Ia through expression of downstream transcription factors like Interferon Regulatory Factor 1 (IRF1) and Nod-like receptor family CARD domain containing 5 (NLRC5), which in turn bind to the *HLA* promoter to induce transcription (15–19). Although the *MR1* gene resides on human chromosome 1, outside the chromosome 6 *HLA* locus (7), these pathways may provide insight into transcriptional regulation of MR1. Recent research links *MR1* expression with disease pathology (e.g. meningeal tuberculosis (20), glioma (21), and COPD (22–24)), although specific mechanisms controlling *MR1* transcription remain unclear.

Here, we investigated the role of IFN γ in stimulating MR1 expression in human airway epithelial cells (AEC). We found IFN γ promotes MR1 transcription, antigen presentation, and MAIT cell responses. While NLRC5 and IRF1 were both important for IFN γ -induced *HLA-A* transcription, NLRC5 was largely dispensable for *MR1* transcription. Finally, we demonstrate that MAIT cells, activated in co-culture with infected AEC, produce sufficient IFN γ to stimulate *MR1* transcription. Taken together, our data support a model in which IFN γ from activated immune cells induces MR1 expression and antigen presentation, leading to greater MAIT cell activation. These results establish an additional level of MR1 regulation, informing our understanding of MAIT cell activation and dysregulation in infection and disease.

Results

MR1 expression increases in infected AECs co-cultured with MAIT cells

First, we asked if co-culture with activated MAIT cells could impact MR1 expression and function in airway epithelial cells (AEC). To address this, we examined *MR1* mRNA expression of primary human AEC co-cultured with the human MAIT cell clone D426G11 alone or in the context of *Streptococcus pneumoniae* (*Sp*) infection.

We noticed significantly increased *MR1* mRNA expression in AEC from healthy donors when infected with *Sp* and cultured with MAIT cells (Figure 1A, Supplementary Figure 1A, Supplementary Table 1). Infection with *Sp* or co-culture with MAIT cells alone did not stimulate a significant response. We replicated this system using a model bronchial epithelial cell line (BEAS-2B cells) infected with *Mycobacterium smegmatis* (*Ms*). Similarly, we observed increased *MR1* expression in the *Ms*-infected BEAS-2B cells co-cultured with MAIT cells, with no impact of either condition alone (Figure 1B, Supplementary Figure 1B, Supplementary Table 1). Using flow cytometry to quantify surface MR1 protein expression, we likewise found increased MR1 expression with both *Ms* infection and MAIT cell co-culture compared to either condition alone (Figure 1C, Supplementary Figure 1C). These data suggest that MAIT cells, when activated by presentation of bacterial antigens, could lead to increased *MR1* mRNA or surface protein expression in the infected cell.

We next asked whether microbial infection is required for this transcriptional increase or if the presence of MR1 ligand alone is sufficient. We treated BEAS-2B cells with either the stimulatory antigen 5-(2-oxopropylideneamino)-6-d-ribitylaminouracil (5-OP-RU) or the non-stimulatory ligand 6-formylpterin (6-FP) and measured expression of MR1. Neither 5-OP-RU nor 6-FP increased *MR1* expression alone (Figures 1D, E). In co-culture with MAIT cells, however, *MR1* mRNA expression increased when BEAS-2B cells were treated with 5-OP-RU, while treatment with 6-FP had no impact on *MR1* expression (Figures 1D, E, Supplementary Figures 1D, E). These data demonstrate that the upregulation of *MR1* mRNA expression requires activation of MAIT cells, and this may be stimulated by antigen presentation alone or bacterial infection.

IFN γ stimulates MR1 expression and antigen presentation

Among the effector molecules produced by activated MAIT cells, IFN γ is well known to stimulate transcription of MHC Class I molecules (15–17). We hypothesized *MR1* expression could be regulated through similar mechanisms, despite the differences in chromosomal location and gene arrangement from classical *HLA* genes. To test if IFN γ alone is sufficient to stimulate *MR1* expression, we treated primary AEC with recombinant human

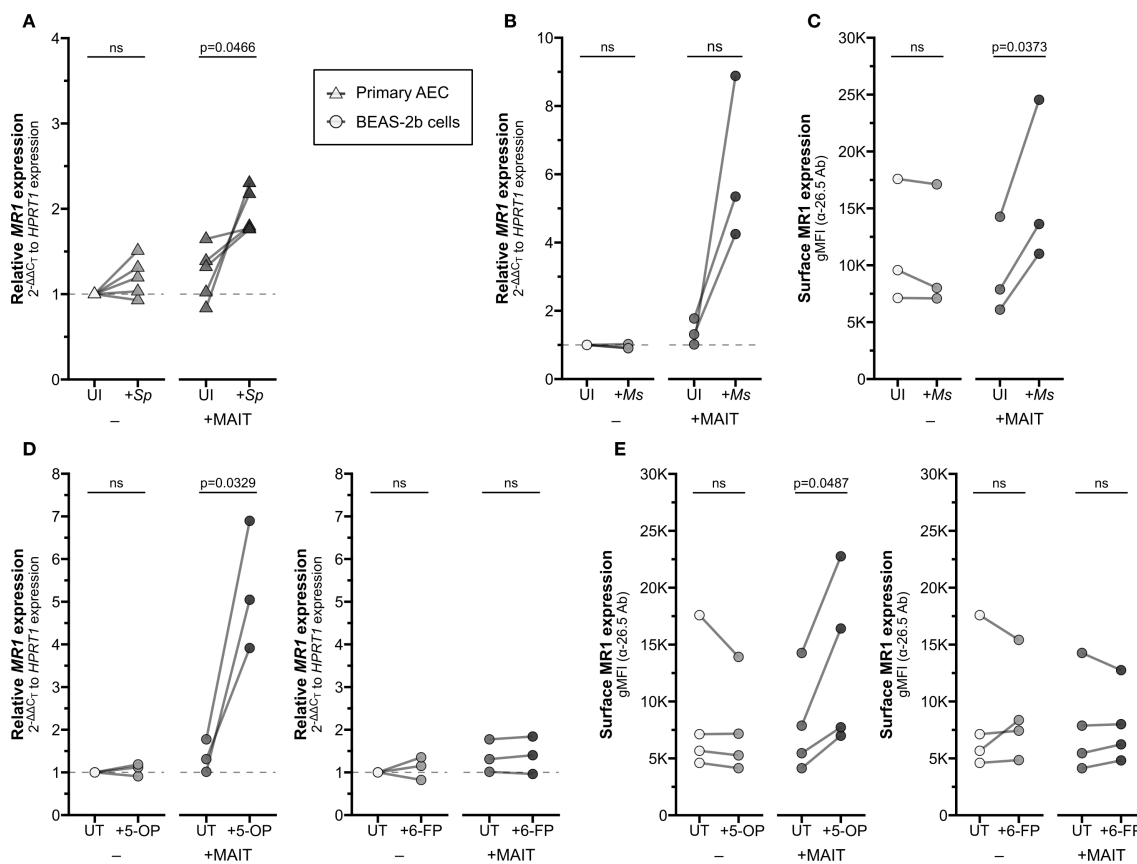


FIGURE 1

Increased MR1 expression following MAIT cell activation. (A) RT-qPCR of RNA isolated from primary human AECs ($n=5$) infected with *S. pneumoniae* (Sp) for one hour and incubated overnight with MAIT cell clone. MR1 expression was calculated relative to HPRT1 expression and uninfected no-MAIT (UI-) controls, paired by individual donor. MR1 (B) mRNA and (C) surface expression of BEAS-2B cells infected with *M. smegmatis* (Ms) for one hour and incubated overnight with MAIT cell clone. (B) RT-qPCR of MR1 expression was calculated relative to HPRT1 expression and UI- control, paired by experimental replicate. (C) Geometric mean fluorescence intensity (gMFI) of surface MR1 stained with α -MR1 26.5 Ab, paired by experimental replicate. MR1 (D) mRNA and (E) surface expression of BEAS-2B cells treated with 5-OP-RU (left, "5-OP") or 6-FP (right) for one hour and incubated overnight with MAIT cell clone. (D) RT-qPCR of MR1 expression was calculated relative to HPRT1 expression and UT- control, paired by experimental replicate. (E) gMFI of surface MR1 stained with α -26.5 Ab, paired by experimental replicate. Pairwise statistical analyses are in [Supplementary Table 1](#). Triangles represent data from primary AEC and circles represent data from BEAS-2B cells. The symbol 'ns' refers to comparisons with p-values < 0.05.

IFN γ . IFN γ treatment significantly increased MR1 transcription (Figure 2A, left). As expected, IFN γ also increased expression of positive control HLA-A (Figure 2A, right). In BEAS-2B cells treated with IFN γ , we also observed significant increases in both MR1 and HLA-A mRNA expression (Figure 2B).

To quantify MR1 protein expression, we measured surface MR1 expression by flow cytometry. We found that IFN γ treatment also significantly increased surface MR1 expression and control MHC-I expression in primary AEC and BEAS-2B cells (Figures 2C, D, Supplementary Figure 1F). This approach does not distinguish between 1) increased surface expression of MR1 proteins due to increased MR1 transcription and translation or 2) increased translocation of existing MR1 molecules and stabilization on the cell surface. To determine if IFN γ signaling impacts post-transcriptional protein stability of MR1, we utilized BEAS-2B cells expressing MR1-GFP under a doxycycline-inducible promoter (11). IFN γ treatment did not increase expression of MR1 mRNA, total MR1-GFP gMFI, or surface MR1 in these doxycycline-treated cells (Figure 3A-C). As expected, IFN γ treatment increased MHC-Ia

surface expression and 6-FP treatment induced significant stabilization of total MR1-GFP protein expression and surface translocation (Figures 3C, D). Together, these data indicate that increased surface MR1 in IFN γ -treated wildtype cells resulted from stimulation of MR1 transcription rather than protein-level impacts.

MR1 antigen presentation and MAIT cell responses are increased in MR1 over-expression systems (10, 11, 25). We next investigated if the IFN γ -dependent increase in MR1 expression similarly enhanced MAIT cell responses to wildtype cells. BEAS-2B cells were treated with IFN γ for 12 hours, thoroughly washed to remove excess soluble IFN γ , then used as antigen-presenting cells in an IFN γ ELISPOT assay to quantify MAIT cell activation. Filtered *M. smegmatis* supernatant was used as the antigen source to avoid potential confounding impacts of IFN γ treatment on bacterial infection. MAIT cell responses to IFN γ pre-treated BEAS-2B cells were significantly increased compared to UT controls (Figure 3E). Therefore, IFN γ treatment is sufficient to stimulate MR1 transcription, leading to increased protein expression and antigen presentation to MAIT cells.

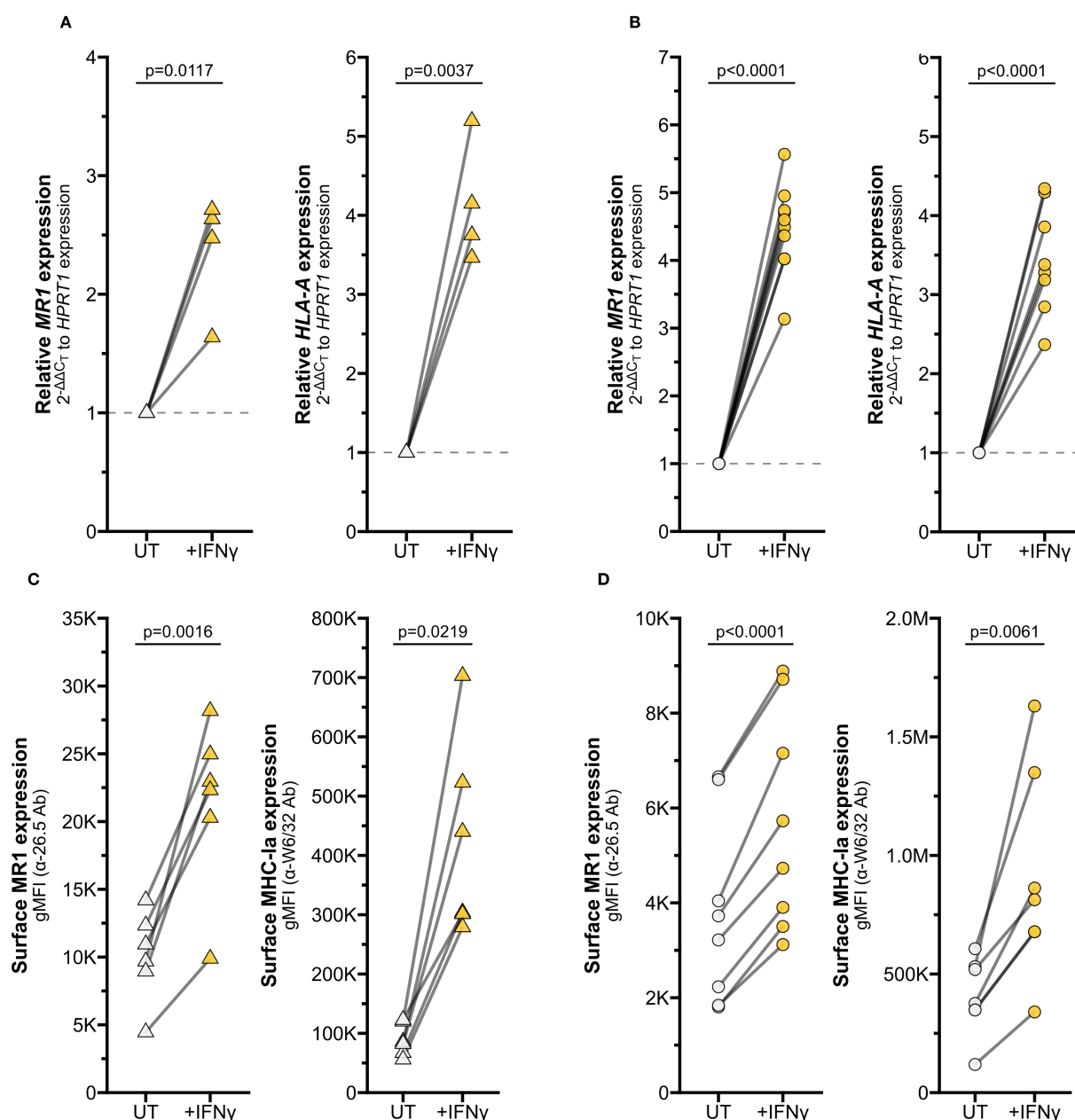


FIGURE 2

IFN γ induces MR1 expression and function. RT-qPCR of (A) primary human AECs or (B) BEAS-2B cells treated with media control (UT) or recombinant IFN γ for 12 hours. *MR1* (left) and *HLA-A* (right) expression were calculated relative to *HPRT1* expression and UT control, paired by individual donor or experimental replicate. Flow cytometry of (C) primary AECs or (D) BEAS-2B cells treated with recombinant IFN γ for 12 hours. gMFI of surface MR1 (left, α -26.5 Ab) and MHC-Ia (right, α -W6/32 Ab) are paired by individual donor or experimental replicate. Pairwise T tests were performed by donor (A, C) or experiment (B, D). Triangles represent data from primary AEC and circles represent data from BEAS-2B cells. Yellow symbols indicate IFN γ treatment.

IFN γ stimulates MR1 transcription via transcription factor IRF1, not NLRC5

Using MHC-Ia transcription pathways as a starting point, we queried the JASPAR CORE 2018 Vertebrates database to determine if the *MR1* promoter contained binding motifs for known IFN γ -induced transcription factors (26–28). We highlighted notable predicted elements on this region, including those common with IFN γ -mediated *HLA* transcription factor

sites (Figure 3F). For example, we found putative binding motifs for IRF1 and members of the NLRC5 enhanceosome complex (18, 19, 29–31). This targeted search suggested that IRF1 and NLRC5 could be of interest in IFN γ -mediated *MR1* transcription.

We first validated that IFN γ signaling induces *IRF1* and *NLRC5* mRNA expression in our cells. Transcripts of both these genes were significantly increased by IFN γ treatment of both primary human AEC and BEAS-2B cells (Figures 3G, H).

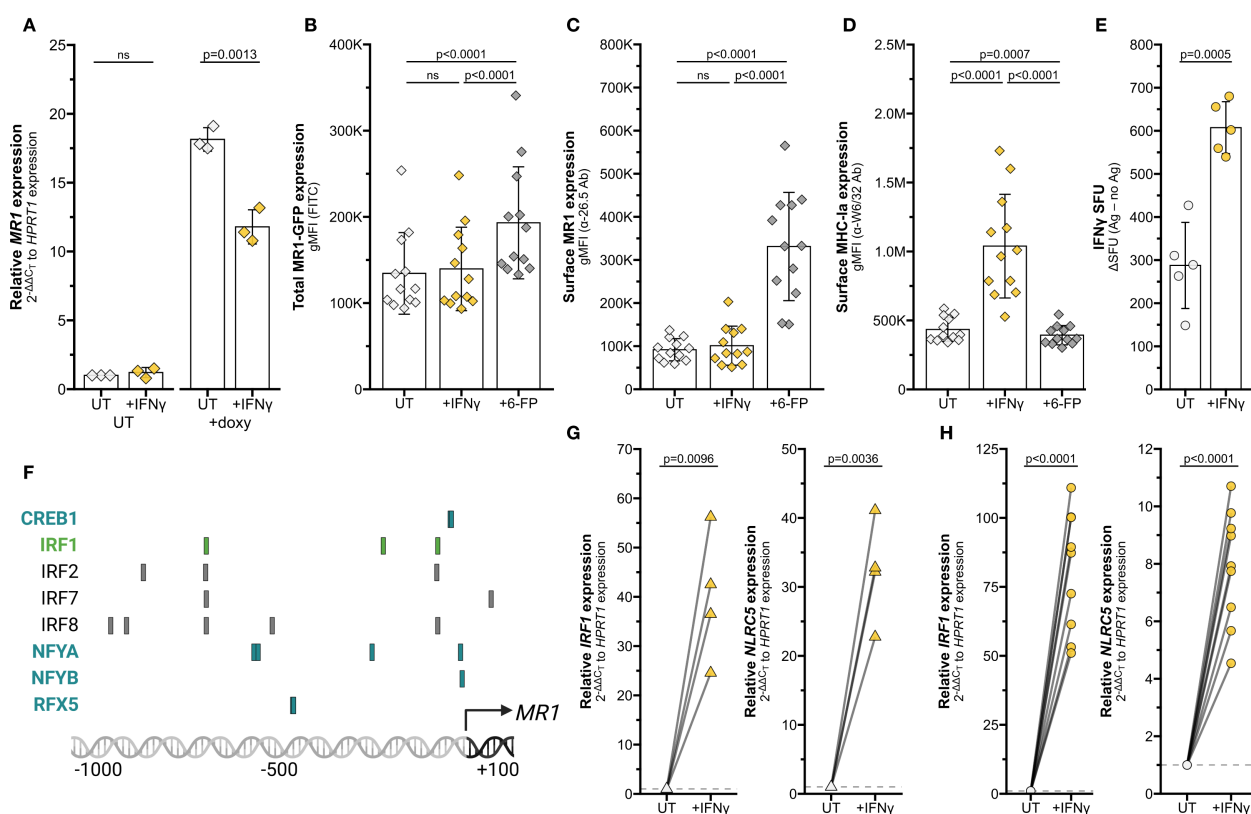


FIGURE 3

Transcriptional stimulation of *MR1* by IFN γ . (A–D) BEAS-2B:doxMR1-GFP cells were treated with doxycycline, IFN γ , and/or 6-FP overnight. (A) *MR1* expression was calculated relative to *HPRT1* expression and UT control, paired by experimental replicate. gMFI of (B) *MR1*-GFP, (C) surface *MR1* α -26.5 stain, and (D) surface MHC-Ia α -W6/32 stain. Data are experimental replicates. (E) ELISPOT of BEAS-2B cells treated with filtered *M. smegmatis* supernatant and MAIT cells. Data points are experimental replicates of no-antigen background-subtracted IFN γ spot-forming units (SFU). Subtracting the background SFU (average 15.6 SFU for UT and 33.7 SFU for IFN γ -treated cells) did not impact statistical significance. (F) Putative transcription factor binding sites were acquired through the Eukaryotic Promoter Database browser using the Search Motif Tool to perform on-the-fly scanning for transcription factor motifs using the FindM tool from the Signal Search Analysis (SSA) Server toolkit (28, 102–104). Highlighted proteins are involved in IRF1- (green) or NLRC5 enhanceosome- (blue) mediated *HLA* transcription. RT-qPCR of (G) primary human AECs or (H) BEAS-2B cells treated with recombinant IFN γ for 12 hours. *IRF1* (left) and *NLRC5* (right) expression were calculated relative to *HPRT1* expression and UT control, paired by individual donor or experimental replicate. Pairwise T tests were performed by experiment (A–E, H) or donor (G). Diamonds represent data from BEAS-2B:doxMR1-GFP cells, triangles represent data from primary AEC, and circles represent data from BEAS-2B cells. Yellow symbols indicate IFN γ treatment.

To determine if IRF1 or NLRC5 are required for the IFN γ -mediated increase in *MR1* transcription, we first used siRNA to knock down the genes separately or together in BEAS-2B cells. IRF1 KD alone significantly decreased IFN γ -stimulated *MR1* mRNA expression compared to missense controls (Figure 4A, Supplementary Table 2). Although IRF1 siRNA significantly reduced *IRF1* expression, we were unable to sufficiently knock down *NLRC5* expression by siRNA (Supplementary Figures 2A, B). Therefore, we generated monoclonal *NLRC5*^{-/-} BEAS-2B cell lines by CRISPR/Cas9. Loss of *NLRC5* did not lead to any significant impact to IFN γ -induced *MR1* mRNA expression (Figure 4B). We did observe a decrease in *HLA-A* expression with *NLRC5* knockout, although not significant (Figure 4C).

We used siRNA to silence IRF1 expression in the Cas9⁺ and *NLRC5*^{-/-} cells (Supplementary Figure 2C). *MR1* expression was significantly impacted by IRF1 knockdown in Cas9⁺ control cells (Figure 4B), agreeing with the previous siRNA results in wildtype BEAS-2B cells. A trend in reduced *MR1* expression was also

observed in two clones of *NLRC5*^{-/-} cells treated with IRF1 siRNA (Figure 4B, Supplementary Figure 2D). Expression of surface *MR1* proteins in IFN γ -treated cells was similarly decreased with IRF1 silencing and unaffected by *NLRC5* knockout (Supplementary Figure 2E). Finally, we used ELISPOT assays to quantify if loss of *NLRC5* and/or IRF1 would impact the IFN γ -stimulated boost in *MR1* antigen presentation to MAIT cells. IRF1 siRNA knockdown significantly reduced MAIT cell responses to both Cas9⁺ cells and *NLRC5*^{-/-} cells (Figure 4D). The missense-treated *NLRC5*^{-/-} cells stimulated similar MAIT cell activity as the Cas9⁺ control cells in response to IFN γ (Figure 4D, Supplementary Table 2). Together, these results indicate that IRF1 expression is required for IFN γ stimulation of *MR1* expression and antigen presentation function, while *NLRC5* does not appear to impact this pathway.

We next used CRISPR/Cas9 knockout to generate monoclonal *IRF1*^{-/-} BEAS-2B cell lines to confirm this finding. IFN γ -mediated stimulation of *MR1* mRNA and *MR1* surface expression was impaired in *IRF1*^{-/-} cells compared to Cas9⁺ cells (Figures 4E,G,

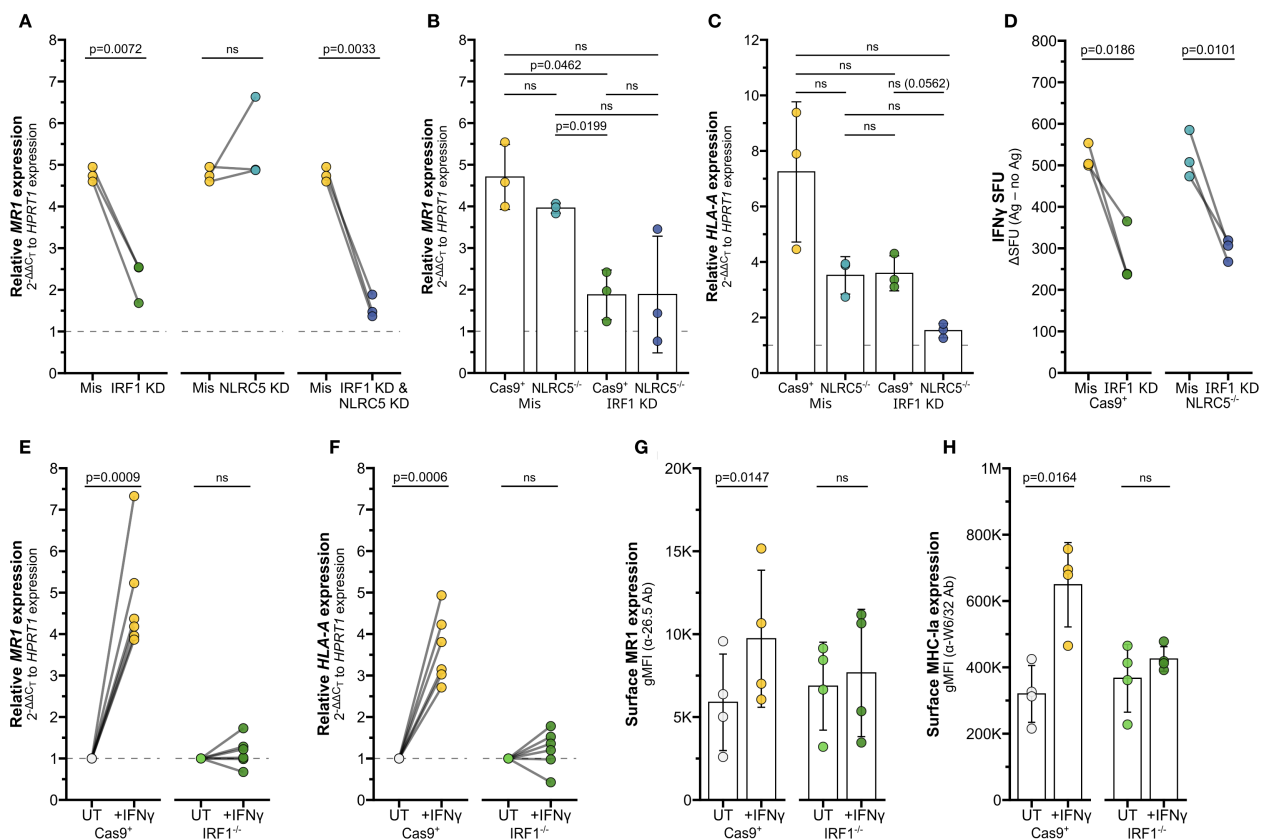


FIGURE 4

IRF1 mediates IFN γ -induced MR1 transcription. (A) RT-qPCR of BEAS-2B cells treated with IRF1, NLRC5, and/or missense siRNA as labeled for 36 hours, then incubated with IFN γ for 12 hours. MR1 expression was calculated relative to *HPRT1* expression and missense UT control, paired by experimental replicate. (B, C) RT-qPCR of Cas9⁺ or NLRC5^{-/-} clone #1 BEAS-2B cells treated with IRF1 or missense siRNA for 36 hours, then incubated with IFN γ for 12 hours. (B) MR1 and (C) HLA-A expression were calculated relative to *HPRT1* expression and Cas9⁺ or NLRC5^{-/-} clone #1 missense UT controls, paired by experimental replicate. (D) Cells in (B, C) were used as antigen-presenting cells in ELISPOT assay, with filtered *M. smegmatis* supernatant as the antigen source. Data points are experimental replicates of Cas9⁺ or NLRC5^{-/-} clone #1 missense control no-antigen background-subtracted IFN γ SFU. Subtracting the background SFU (averages: Cas9⁺ missense 31.3 SFU, Cas9⁺ IRF1 KD 18.3 SFU, NLRC5^{-/-} missense 22.0 SFU, NLRC5^{-/-} IRF1 KD 13.9 SFU) did not impact statistical significance. (E, F) RT-qPCR of Cas9⁺ or IRF1^{-/-} clone #2 BEAS-2B cells treated with IFN γ for 12 hours. (E) MR1 and (F) HLA-A expression were calculated relative to *HPRT1* expression and Cas9⁺ or IRF1^{-/-} clone #2 UT controls, paired by experimental replicate. (G, H) Flow cytometry of Cas9⁺ or IRF1^{-/-} clone #1 BEAS-2B cells treated with IFN γ for 12 hours. gMFI of (G) surface MR1 (α-26.5 Ab) and (H) MHC-Ia (α-W6/32 Ab) are paired by experimental replicate. Statistical analyses are in Supplementary Table 2. Yellow symbols indicate IFN γ treatment alone. For visual clarity, silencing of IRF1 (green), NLRC5 (teal), or both (dark blue) are also indicated. In (E-H), light green distinguishes media control IRF1^{-/-} cells from IFN γ -treated IRF1^{-/-} cells (dark green). The symbol 'ns' refers to comparisons with p-values < 0.05.

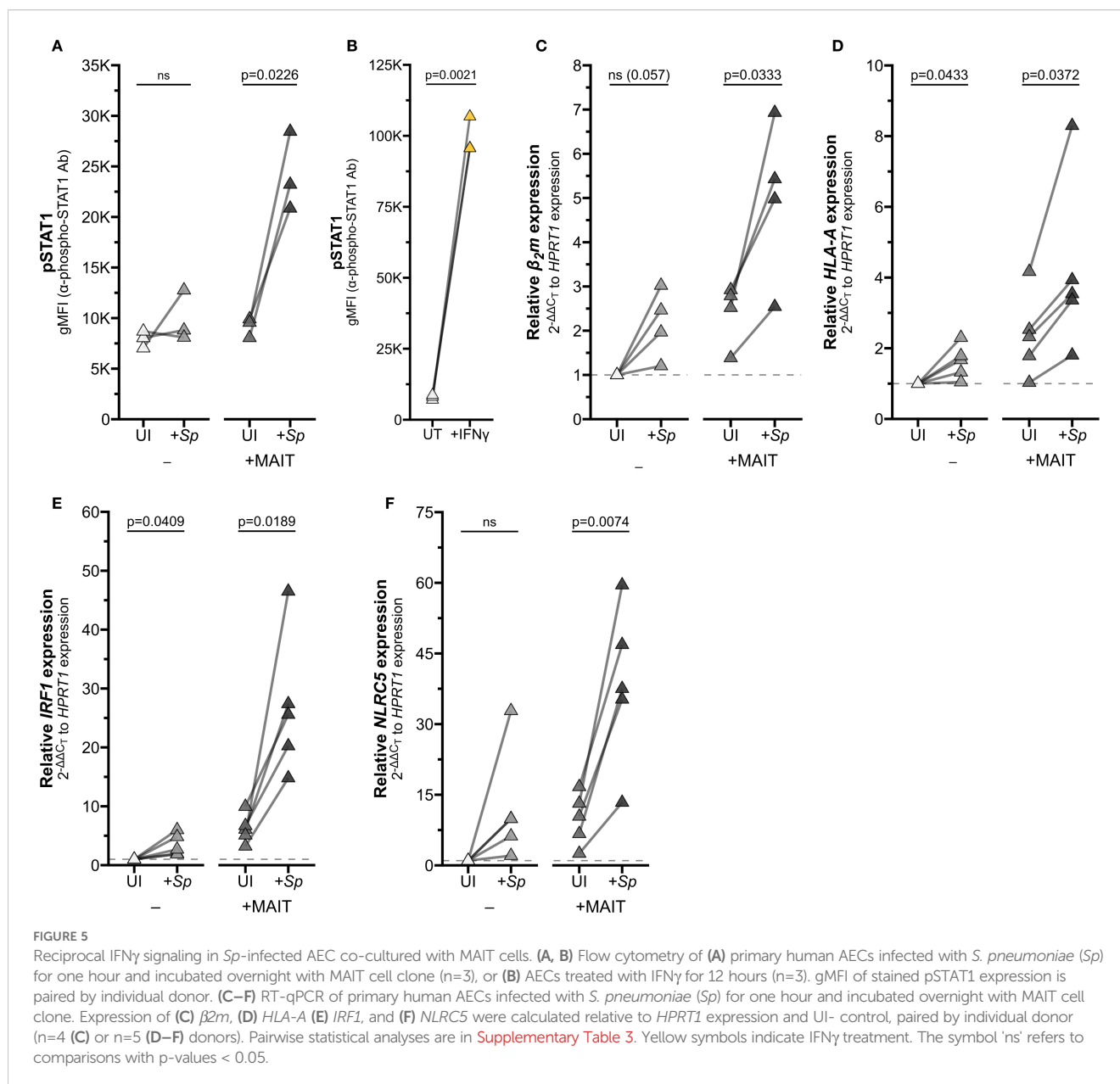
Supplementary Figures 2F, G). Expression of HLA-A mRNA or MHC-Ia surface proteins were likewise impaired in the IRF1^{-/-} cells following IFN γ treatment (Figures 4F,H; Supplementary Figure 2G, right). Together the siRNA knockdown and CRISPR knockout results indicate that IRF1 mediates the IFN γ signaling pathway leading to MR1 transcription, surface expression, and antigen presentation, likely through mechanisms independent of the NLRC5 enhanceosome.

MAIT cells produce sufficient IFN γ to induce MR1 transcription pathways

To validate this mechanism in a physiologically relevant system, we returned to our co-culture experiments. We first demonstrated that co-culture of infected AEC with MAIT cells led to upregulation

of IFN γ -stimulated pathways by staining phosphorylated STAT1 (pSTAT1). Ligation of the IFNGR activates Janus kinase (JAK) dimer 1/2, which in turn phosphorylates STAT1 (17, 32). As expected, pSTAT1 staining was significantly increased in AEC infected with *S. pneumoniae* and co-cultured with MAIT cells, along with AEC treated with recombinant IFN γ (Figures 5A, B, Supplementary Figure 3A, Supplementary Table 3).

To confirm that activation of MAIT cells in co-culture is sufficient to drive IFN γ signaling, we next assessed expression of IFN γ -stimulated genes. Co-culture of *Sp*-infected primary AEC with MAIT cells significantly induced expression of HLA-A, *B2m*, *IRF1*, and *NLRC5* (Figures 5C-F; Supplementary Figures 3B-D, G; Supplementary Table 3). We observed increased expression of these genes in AEC incubated with either *Sp* or MAIT cells alone, which may indicate the contribution of other inflammatory signaling pathways in AEC. However, the combined co-culture induced



significantly greater expression for almost all genes, pointing to the role of Ag-induced MAIT cell activation in driving inflammatory gene expression.

BEAS-2B infected with *Ms* significantly induced expression of *HLA-A* and *IRF1* only in combination with MAIT cell co-culture (Figures 6A, C; [Supplementary Figures 3E, H](#); [Supplementary Table 3](#)). We next used 5-OP-RU as the antigen source to confirm that MR1 antigen presentation alone is sufficient to stimulate MAIT cell IFN γ production and subsequent inflammatory gene expression, absent other microbial stimuli. As expected, *HLA-A* and *IRF1* expression were significantly increased in 5-OP-RU-treated BEAS-2B cells when MAIT cells were present, but not stimulated by treatment with 5-OP-RU alone or MAIT cell co-culture with untreated BEAS-2B cells (Figures 6B, D; [Supplementary Figures 3F, I](#); [Supplementary Table 3](#)). Non-stimulatory presentation of 6-FP

ligands failed to significantly induce either gene alone or in combination with MAIT cells. Together, these results indicate that activated MAIT cells produce sufficient IFN γ to stimulate expression of downstream genes.

Finally, we used our *IRF1*^{-/-} cells in this co-culture setting to demonstrate the role of IFN γ in mediating MR1 expression, antigen presentation, and MAIT cell activation. *IRF1*^{-/-} cells infected with *Ms* and co-cultured with MAIT cells failed to exhibit the increase in *MR1* expression seen in the Cas9⁺ control cells (Figure 6E, [Supplementary Table 4](#)). We then used exogenous 5-OP-RU treatment to directly test the role of *IRF1* following MR1-dependent MAIT cell activation. *MR1* transcription was enhanced in Cas9⁺ cells with 5-OP-RU and MAIT cell co-culture, confirming that TCR-stimulated MAIT cells directly led to increased *MR1* transcription (Figure 6F, [Supplementary Table 4](#)). In contrast, the

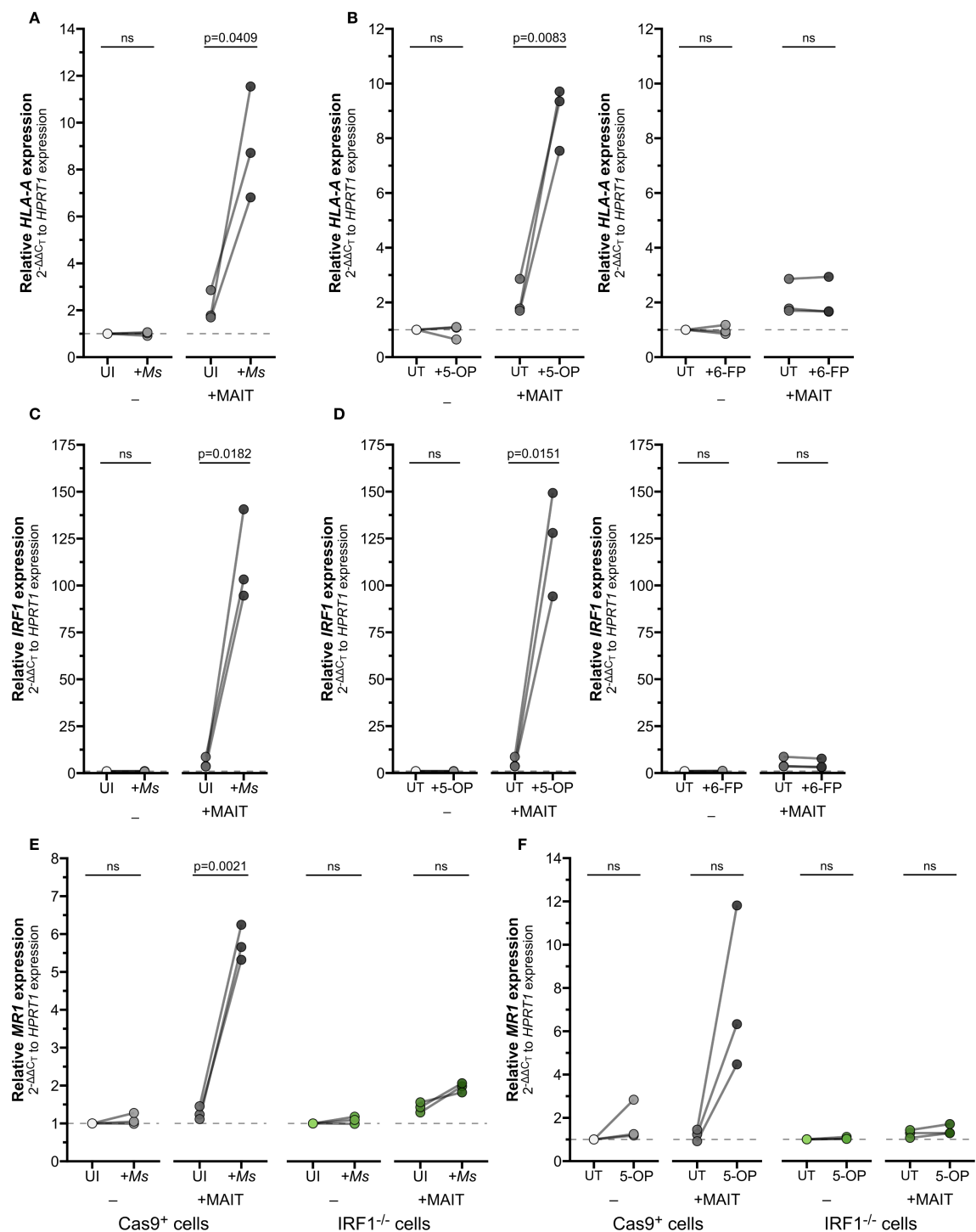


FIGURE 6
IFN γ produced by activated MAIT cells drives IRF1-dependent *MR1* transcription. **(A–D)** RT-qPCR of wildtype BEAS-2B cells treated as indicated below. Gene expression was calculated relative to *HPRT1* expression and UI- or UT- controls, paired by experiment. **(A)** HLA-A and **(C)** IRF1 expression of BEAS-2B cells infected with *M. smegmatis* (Ms) for one hour and incubated overnight with MAIT cell clone. **(B)** HLA-A and **(D)** IRF1 expression of BEAS-2B cells treated with 5-OP-RU (left, “5-OP”) or 6-FP (right) for one hour and incubated overnight with MAIT cell clone. **(E, F)** RT-qPCR of Cas9 $^{+}$ or IRF1 $^{-/-}$ clone #1 BEAS-2B cells **(E)** infected with *M. smegmatis* or **(F)** treated with 5-OP-RU for one hour, then incubated overnight with MAIT cell clone. *MR1* expression was calculated relative to *HPRT1* expression and Cas9 $^{+}$ or IRF1 $^{-/-}$ UT- controls, paired by experimental replicate. Pairwise statistical analyses are in [Supplementary Table 4](#). In **(E, F)**, green symbols distinguish IRF1 $^{-/-}$ cells from Cas9 $^{+}$ cells (gray) as labeled. The symbol ‘ns’ refers to comparisons with p-values < 0.05.

IRF1^{-/-} cells treated with 5-OP-RU did not express greater *MR1* transcripts in co-culture, confirming the importance of IRF1 in this pathway.

IFN γ and IFN β stimulate MR1 transcription by distinct mechanisms

Our data have shown thus far that IFN γ stimulates *MR1* transcription; however other inflammatory cytokines can also induce transcription. Type I interferons like IFN β stimulate IRF1 and NLRC5 to induce MHC-Ia transcription (15–19, 33, 34). Activated MAIT cells may also produce TNF α and IL-17, which have been demonstrated to stimulate transcription of inflammatory genes including *IRF1* and *HLA-A* (35–37). To assess whether the IFN γ -induced increase in *MR1* transcription is representative of general inflammatory signaling mechanisms or specific to IFN γ stimulus, we treated BEAS-2B cells with recombinant human inflammatory cytokines IFN β , IFN γ , IFN λ , TNF α , and IL-17. Of these cytokines, only IFN β and IFN γ elicited a significant increase in *MR1* transcription compared to untreated controls (Figures 7A, B, Supplementary Table 5).

Interestingly, the IFN γ -mediated increase in MR1 expression was significantly greater than the increase due to IFN β treatment, while expression of *HLA-A* and *B2m* were similarly induced by both IFN γ and IFN β treatment (Figures 7A–C, Supplementary Figure 4, Supplementary Table 5). We further investigated the role of type I and II IFNs in mediating expression of MR1 and MHC-Ia. While both IFN β and IFN γ increased surface expression of MHC-Ia, only IFN γ led to a significant increase in surface MR1 protein expression (Figure 7D). BEAS-2B cells pre-treated with IFN γ induced significantly greater MAIT cell responses to *Ms* infection than control UT cells, while IFN β pre-treatment did not generate a significantly different dose-response curve (Figure 7E). These data indicate that IFN γ plays the largest role in stimulating MR1 expression and function.

We quantified *IRF1* and *NLRC5* transcripts to further explore how MR1 and MHC-I expression are differentially stimulated by IFN β and IFN γ . Although both IFN γ and IFN β increased *IRF1* expression, the relative fold change was significantly greater with IFN γ than IFN β (Figure 7F, Supplementary Figure 4, Supplementary Table 5). Both interferons induced significant increases in *NLRC5* and *B2m* expression (Figures 7G, H, Supplementary Figure 4, Supplementary Table 5). In light of our finding that IFN γ stimulates *MR1* transcription through IRF1 and not NLRC5, this magnitude of *IRF1* induction may relate to the specific induction of MR1 surface expression and antigen presentation by IFN γ and not IFN β . Further, these results indicate that transcription of *MR1* and *HLA* occur via distinct IFN γ -stimulated mechanisms.

Together, our data support a feed-forward model of inflammatory signaling (Figure 8). MR1 antigen presentation by an infected cell activates a MAIT cell to release IFN γ , which then acts on the airway epithelial cell to stimulate *IRF1* expression through a pSTAT1 pathway. IRF1 then binds to the *MR1*

promoter to induce *MR1* transcription, leading to more MR1 protein available for antigen presentation and subsequent MAIT cell activation.

Discussion

MAIT cells are key components of early infection responses. The variety of pathogens producing MR1 antigens and rapid MAIT cell effector function poise MAIT cells to bridge innate and adaptive immune responses. Strict regulation of MAIT cell activation is required to prevent inflammatory damage. Research over the past decade has defined many complementary pathways regulating MR1 intracellular localization, antigen binding, surface translocation, and protein recycling (12–14). These studies affirm that defining the mechanisms that regulate MR1 is critical to understanding regulation of MAIT cells themselves. Only recently have we begun to appreciate the role of epigenetic regulation in controlling MR1 expression and antigen presentation. Studies of *MR1* DNA methylation and RNA expression suggest that *MR1* transcription is increased during infection (38–41), although effector molecules from herpes simplex viruses were shown to degrade *MR1* transcripts (42–44). Altered epigenetic regulation of *MR1* in respiratory inflammation (22–24, 45) and cancer (21, 46) illustrate the complex interplay of activation and repression signals in these diseases.

Here, we demonstrate for the first time that IFN γ stimulates *MR1* transcription. We investigated the role of two IFN γ -stimulated transcription factors, IRF1 and NLRC5, in regulating *MR1* transcription. *In-silico* analysis of the *MR1* promoter revealed potential binding sites for IRF1 and components of the NLRC5 enhanceosome (18, 19). Using both siRNA knockdown and CRISPR/Cas9 knockout systems, we observed that IFN γ -induced *MR1* transcription was dependent on IRF1, but not NLRC5. Treatment with IFN γ failed to increase MR1 surface expression and antigen presentation in IRF1^{-/-} cells. These results pointed to IRF1 as the primary driver of IFN γ -induced *MR1* transcription in our experimental conditions. The rapid increase in *IRF1* expression after IFN γ treatment is consistent with established models of IRF1 kinetics (18, 47). Recently, Rosain and colleagues performed a comprehensive characterization of two young patients with IRF1-inactivating mutations (48). *MR1* was among the genes upregulated in primary fibroblasts from healthy controls after 8 hours of IFN γ treatment, while *MR1* was not upregulated in IFN γ -treated fibroblasts with IRF1-inactivation, STAT1-deficiency, or loss of IFNGR1/2 (48). Our co-culture experiments showed that antigen-activated MAIT cells produced sufficient IFN γ to induce *IRF1* expression in both primary AEC and BEAS-2B cells. In similar conditions with *Ms* infection or 5-OP-RU treatment and MAIT cell co-culture, IRF1^{-/-} cells failed to replicate the increase in *MR1* mRNA expression seen with control Cas9⁺ cells. We therefore concluded that MAIT cell activation acts through IRF1 to promote *MR1* transcription. The case study of IRF1-deficient patients observed only slightly lower blood MAIT cell frequencies in one individual (48). However, both experienced persistent early

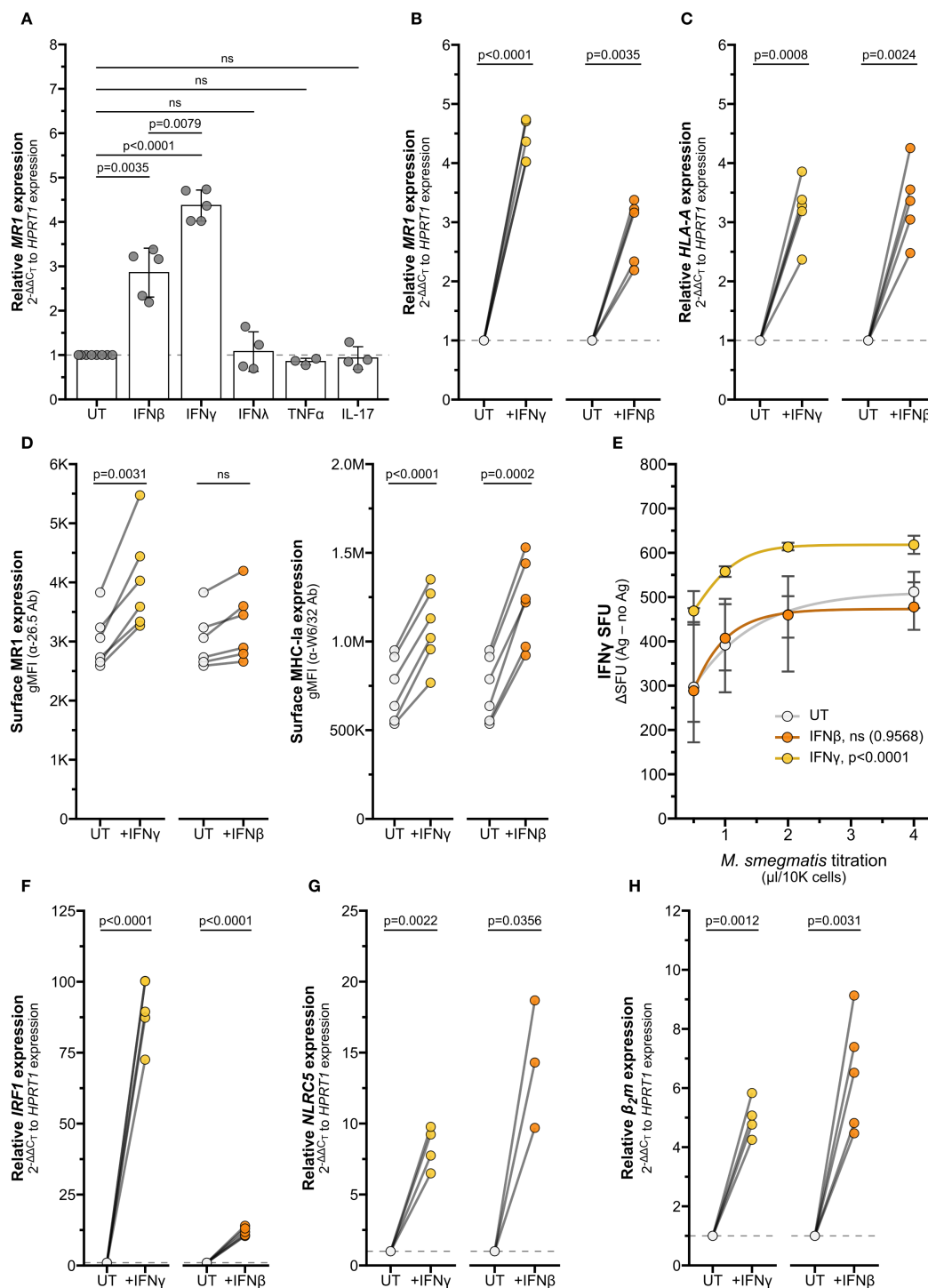


FIGURE 7

Interferons stimulate MR1 and MHC-Ia transcription through different pathways. (A) RT-qPCR of wildtype BEAS-2B cells treated with recombinant human cytokines for 12 hours. MR1 expression was calculated relative to HPRT1 expression and UT controls. (B, C, F–H) RT-qPCR of BEAS-2B cells treated with IFN γ or IFN β for 12 hours. Expression of (B) MR1, (C) HLA-A (F) IRF1, (G) NLRC5, and (H) β_2m were calculated relative to HPRT1 and UT control, paired by experiment. (D) Flow cytometry of BEAS-2B cells treated with IFN γ or IFN β for 12 hours. gMFI of surface MR1 (left, α -26.5 Ab) and MHC-Ia (right, α -W6/32 Ab) are paired by experimental replicate. (E) ELISPOT of BEAS-2B cells treated with IFN γ or IFN β for 12 hours, infected with a titration of *M. smegmatis* for one hour, then incubated with MAIT cells overnight. Data points are average SFU of no-antigen background-subtracted IFN γ SFU. Nonlinear regression agonist response curves were computed in GraphPad Prism 10.4.0 and analyzed with extra sum-of-squares F test to compare with UT control. Statistical analyses are in [Supplementary Table 5](#). Yellow symbols indicate IFN γ treatment, orange symbols indicate IFN β treatment, and white symbols indicate UT controls. The symbol 'ns' refers to comparisons with p-values < 0.05.

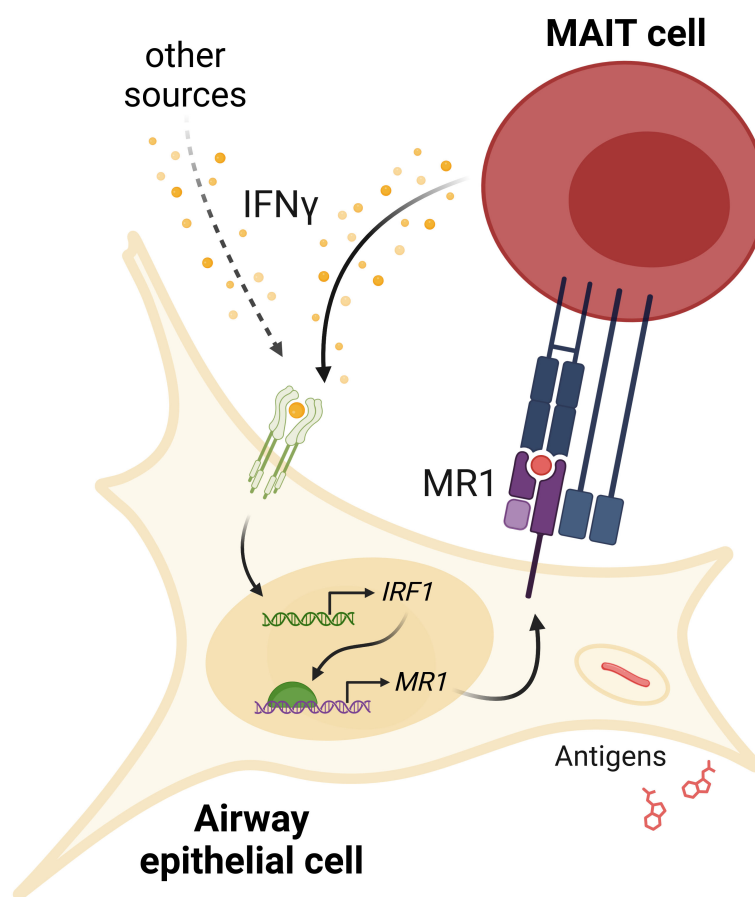


FIGURE 8

IFN γ signaling induces MR1 expression and MAIT cell activation. IFN γ from MAIT cells or other cellular sources induces *IRF1* expression and subsequent increase in *MR1* transcription. Increased MR1 expression and antigen presentation enhances MAIT cell responses to antigens from exogenous sources or pathogens like *S. pneumoniae* or *M. smegmatis*.

childhood infections from weakly-infectious *Mycobacterium avium* and/or breakthrough infection from the BCG vaccine, consistent with impaired IFN γ immunity (48). Although many factors may contribute to reduced immune function in these individuals, MAIT cells have been tightly linked to anti-mycobacterial immunity (49, 50). A case study of a T-bet-deficient individual with very low MAIT cells underscored the specific importance of IFN γ production by innate-like lymphocytes in controlling *Mtb* infection (51). It is therefore possible that loss of IRF1 function could foster mycobacterial susceptibility through weaker induction of *MR1* transcription and delayed MAIT cell responses. Directly characterizing the dynamics of *MR1* expression in these individuals would shed light on this hypothesis.

We were surprised to observe that NLRC5 was not required for the IFN γ -induced increase in MR1 expression or antigen presentation, given the importance of NLRC5 in mediating *HLA* transcription (15, 16, 19). Although MR1 is an MHC-I-like molecule and they share broad structural homology, the genes reside on different chromosomes and have distinct promoter features (7). We confirmed that *HLA-A* expression was reduced in NLRC5^{-/-} cells. Since IRF1 can also induce *NLRC5* transcription,

we considered whether IRF1 and NLRC5 play a synergistic role in regulating MR1 expression. While the combined loss of IRF1 and NLRC5 reduces Class I mRNA expression, we saw no further impact to MR1 expression or antigen presentation. These results prompted us to explore how MHC-Ia and MR1 transcription signaling pathways diverge. Both type I and type II IFNs are known to stimulate expression of IRF1 and MHC-Ia (15–18, 33, 34). We explored whether IFN β might induce *MR1* transcription similarly to IFN γ . Although both interferons increased *MR1* mRNA expression, IFN γ stimulated a significantly greater increase in *MR1* transcription than IFN β . Furthermore, only IFN γ induced MR1 surface protein expression and antigen presentation to MAIT cells. Previously, Ussher et al. demonstrated that MAIT cell responses to fixed intact *E. coli* were significantly increased when THP1 cells were incubated with either IFN α or IFN γ overnight (52). This type I IFN increase does not match our results. However, several other groups observed that directly stimulating MAIT cells with IFN β or IFN α led to increased TCR-dependent and -independent MAIT cell responses to influenza virus and *Klebsiella pneumoniae* infection (53–55). Therefore, type I interferon-induced signaling within MAIT cells may be the primary driver of the observed increase in

MAIT cell responses (52). Type III interferons signal through similar pathways as type I interferons and are critical to some mucosal inflammatory responses (56). We failed to measure any notable increase in *MR1* or *HLA-A* expression following IFN λ treatment. We observed significant increases in *IRF1* and $\beta 2m$ expression with IFN λ ; however, these increases were significantly weaker than any IFN γ - or IFN β -induced *IRF1* or $\beta 2m$ expression. This result is consistent with research from Forero et al., who found that IFN λ primarily induces tissue repair pathways and fails to stimulate *IRF1* expression (57, 58).

Others have shown that IFN γ , not IFN β , is the primary driver of *IRF1* expression (48). We quantified the relative increase in *IRF1* transcripts following stimulation by IFN γ or IFN β and likewise observed the increase in *IRF1* expression was significantly higher with IFN γ compared to IFN β . In contrast, both interferons led to similar ranges of *NLRC5* expression. One possible model of this data suggests that IFN γ signaling induces sufficient *IRF1* expression to promote *MR1* transcription. IFN β induces less *IRF1* transcription, leading to expression of *NLRC5*, *MHC-Ia*, and $\beta 2m$, but not enough to stimulate *MR1* expression and function. It is tempting to speculate that the specificity of *MR1* stimulation by IFN γ may help to compartmentalize immune responses to innate signaling and prevent simultaneous overstimulation of both *MHC-Ia* and *MR1*.

We also observed a slight increase in *IRF1* expression following TNF α treatment, yet no increase in *HLA-A*, $\beta 2m$, or *MR1* expression. It was surprising that TNF α did not stimulate any significant increase in *IRF1*-induced genes. The TNF receptor-associated factor 6 (TRAF6) works with cellular inhibitor of apoptosis 2 (cIAP2) to K63 ubiquitinate *IRF1*, leading to increased function and blocking K48 ubiquitin-mediated *IRF1* proteasomal degradation (18, 47, 59–63). This process, however, functions in concert with Src-family kinases following TLR4, TLR7/9, or IL-1 signaling (18, 47, 61–65). It is possible that this signaling occurred in our experimental condition with primary AEC infected with TLR4 ligand-producing *S. pneumoniae*, since we observed increases in *IRF1* and *HLA-A* expression with or without MAIT cells. However, we also observed *IRF1*-dependent *MR1* expression following 5-OP-RU-induced MAIT cell activation, in which circumstance TLR signaling was likely inactive. Exploring the role of innate sensors and non-interferon cytokines in modulating *IRF1* activity may reveal additional factors that can induce or repress *MR1* expression.

In co-culture settings, IFN γ -stimulated increases in *MR1* expression resulted in greater *MR1* antigen presentation and subsequent MAIT cell activation. MAIT cells activated by *MR1* antigen presentation produced sufficient IFN γ to promote *MR1* transcription and increased *MR1* surface expression. This feed-forward signaling model would support the function of MAIT cells in immune surveillance and early infection response. Robust stimulation of *MR1*-dependent MAIT cell activation could be beneficial during infection onset, allowing minimal antigen stimulus to generate expansive and rapid proinflammatory activity. MAIT cell effector functions are well-established in

priming myeloid cells and recruiting CD4⁺ and CD8⁺ T cells (66, 67). In addition to MAIT cells, a number of other cells could produce IFN γ and initiate this feed-forward loop. Local IFN γ production by professional antigen-presenting cells has been observed in infection contexts; for example, alveolar macrophages produce IFN γ during *M. tuberculosis* infection (68, 69). NK cells, ILC, airway-resident lymphocytes, and circulating lymphocytes are also known to make IFN γ in response to a variety of inflammatory stimuli as well (70, 71). Beyond cytokine signaling, TLRs and C-type lectin receptors can also stimulate expression of *IRF1* and *IRF1*-inducible genes, suggesting that *IRF1* inflammation could be mediated in response to non-interferon stimuli (72). Lepore et al. found that tumor cell self-antigens were presented by *MR1* to non-MAIT *MR1*-restricted T (*MR1T*) cells (73, 74). In this context, *MR1T* cell activation could induce *MR1* expression and stimulate immune responses despite the absence of TLR ligands or other foreign molecules. IFN γ and *IRF1* signaling through any of these sources could stimulate *MR1* expression and activate MAIT cells, leading to enhanced inflammatory responses.

However, dysregulation of this feed-forward loop could also lead to MAIT cell-caused pathology. Inappropriate MAIT cell activation is implicated in autoimmune diseases and chronic inflammation (75, 76). Overproduction of IFN γ contributes to inflammatory lung damage and can stimulate further IFN γ production by alveolar macrophages (68, 77–79). CD8⁺ T cell infiltration is associated with increased disease severity in chronic obstructive pulmonary disease (COPD), and IFN γ signaling is increased in the lungs of COPD patients (79–82). *MR1* transcriptional expression was increased in AEC (22) and PBMC (24) from infected COPD donors. It is possible that the increased IFN γ present in the COPD airway environment could stimulate *MR1* expression and lead to increased MAIT cell activity via the proposed feed-forward signaling loop. IFN γ signaling in response to inappropriate stimuli (e.g. antigens from commensal microbes) could also induce *MR1* transcription and promote ligand-driven MAIT cell inflammatory pathology.

Given the potential for inflammatory damage due to this feed-forward loop, we hypothesize that a dampening mechanism exists to turn off this pathway. Constantin et al. revealed a potential role for ERK1/2 kinases in suppressing *MR1* expression in melanoma, indicating repression mechanisms can modulate *MR1* transcription (46). Specifically, they found the transcription factor ELF1 binds to the *MR1* promoter to stimulate *MR1* expression. ERK1/2, members of the MAPK/MEK signaling cascade, inhibited ELF1 function and subsequent *MR1* transcription. The authors suggested ELF1 inhibition may occur through post-translational modification performed by a downstream intermediary protein (46). Signaling through MEK/ERK was recently demonstrated to inhibit *IRF1* expression and activity in TLR-stimulated macrophages; however, ELF1 mediates antiviral activity in airway epithelial cells independent of interferon and *IRF1* transcriptional activity (83, 84). Multiple distinct mechanisms of *MR1* transcriptional activation could serve several functions: flexible induction of *MR1* expression and function in the context of distinct stimuli, complementary activation to rapidly

enhance MAIT cell responses, and/or as a checkpoint requiring a secondary signal to prevent overactivation. It is well-documented that pathogens target IFN γ signaling and MHC transcription mechanisms to evade immune recognition (34, 85, 86). Several MHC-Ia post-transcriptional repression mechanisms have been identified, including through IRF1 degradation or downregulation of NLRC5 expression (16, 87, 88). In human fibroblasts, *MR1* transcripts were degraded by an RNase protein from herpes simplex virus types 1 and 2, although this mechanism was not specific to *MR1* (42, 43). A greater understanding of how *MR1* transcription is regulated could shed light on these host-pathogen dynamics.

Put together, this work demonstrates that IFN γ signaling stimulates *MR1* transcription, surface expression, and antigen presentation. While we limited our study to airway epithelial cells infected with respiratory pathogens, our model of IFN γ -induced *MR1* transcription may raise intriguing questions outside of this context. Given the variety in baseline *MR1* expression across cell types and tissues (89, 90), exploration of this pathway in additional cells could shed light on *MR1* function within these organs. Furthermore, MAIT cells and MRIT cells play key roles in cancer responses and tissue repair (5, 91). Understanding the mechanisms of *MR1* transcriptional regulation may provide insights into broader immune signaling networks and better inform our knowledge of the roles *MR1* and MAIT cells play in infection and inflammatory diseases.

Materials & methods

Human subjects

This study was conducted according to the principles expressed in the Declaration of Helsinki. Study participants, protocols and consent forms were approved by Oregon Health & Science University Institutional Review Board (IRB00000186). Written and informed consent was obtained from all donors. Human participants are not directly involved in the study. Healthy adults were recruited from among employees at Oregon Health & Science University as previously described to obtain human serum (92).

Cells and bacteria

Primary airway epithelial cells (AEC) were purchased from Lonza Biosciences or harvested from deceased human donor lung tissue through the Cascade Alliance (formerly Pacific Northwest Transplant Bank) as previously described (22, 93). The healthy donor AEC from (22) were likewise grown in Bronchial Epithelial Growth Media ("BEGM", CC-3170) and harvested using ReagentPack Subculture reagents (CC-5034) per manufacturer's protocols (Lonza).

The BEAS-2B bronchial epithelial cell line (CRL-9609, American Type Culture Collection) was grown in DMEM medium (Gibco) supplemented with L-glutamine (25030164, Life Technologies) and 10% heat-inactivated fetal bovine serum ("DMEM-FBS"). BEAS-2B cells overexpressing *MR1*-GFP under a tetracycline-inducible promoter ("BEAS-2B:doxMR1-GFP") (11)

were similarly cultured in DMEM-FBS. Expression of *MR1*-GFP was induced with doxycycline for 16 hours prior to harvest. BEAS-2B cells stably expressing Cas9 (94) were grown in DMEM-FBS and used to generate CRISPR knockouts.

The *MR1*-restricted T cell clone (D426G11) was generated and expanded in RPMI medium (Gibco) supplemented with L-glutamine and 10% heat-inactivated human serum ("RPMI-HuS") as previously described (2, 92).

Streptococcus pneumoniae (95) and *Mycobacterium smegmatis* Mc (2)155 (ATCC) were grown as described in the supplement of (22) and used from frozen stocks. At late log phase, *M. smegmatis* were pelleted and the supernatant was passed through a syringe-driven 0.22 μ m filter and frozen for use as antigen in ELISPOT assays.

Generation of stable CRISPR/Cas9 IRF1 or NLRC5 knockout BEAS-2B cells

We generated IRF1^{-/-} and NLRC5^{-/-} CRISPR knockout BEAS-2B cells as previously described (94). Early passage Cas9⁺ BEAS-2B cells were transduced with sgRNA constructs targeting IRF1 (CRISPR845545_LV, ThermoFisher) or NLRC5 (CRISPR1120312_LV, ThermoFisher) in the presence of 200 μ g Polybrene (Sigma). Following puromycin selection, monoclonal populations were produced by limiting dilution and screened by Western blot or ELISPOT. We validated genomic editing by Sanger sequencing. DNA was isolated from control Cas9⁺, IRF1^{-/-}, and NLRC5^{-/-} BEAS-2B clones using the QIAamp DNA Micro Kit (Qiagen) and amplified by PCR. The OHSU Vollum Institute DNA Sequencing Core performed Sanger sequencing and the resulting sequences were analyzed by TIDE (96) and ICE (97).

Reagents and antibodies

6-formylpterin (6-FP, Schirck's Laboratories) was suspended in 0.01 M NaOH and used at a final concentration of 100 μ M. 5-(2-oxopropylideneamino)-6-d-ribitylamino-uracil (5-OP-RU) was freshly prepared from equal volumes of 32 mM 5-amino-6-d-ribitylamino-uracil (5-A-RU)*HCl (OHSU Medicinal Chemistry Core) (98) and 650 mM methylglyoxal (Sigma) exactly following the second method described in (94) and used at a final concentration of 500 μ M. Phytohemagglutinin PHA-L (L4144 Sigma) was suspended in RPMI-HuS and used at 1 μ g/well. Doxycycline (Sigma) was suspended in sterile water and used at 2 μ g/ml.

Recombinant human cytokines were reconstituted in sterile water and supplemented with bovine serum albumin as per manufacturer recommendations. Final concentrations used were: 66 ng/ml IFN γ (R&D Systems 285-IF-100), 66 ng/ml IFN β (R&D Systems 8199-IF-010), 132 ng/ml IFN λ (PeproTech 300-02K), 66 ng/ml TNF α (R&D Systems 10291-TA-050), and 66 ng/ml IL-17 (PeproTech 200-17). Cells were treated with cytokines for 12 hours unless otherwise noted.

Antibodies used for ELISPOT assays: α -IFN γ (1-D1K, Mabtech) and alkaline phosphatase-conjugated secondary

antibody (7-B6-1-ALP, Mabtech). Antibodies used for Western blot: α -IRF1 (D5E4, Cell Signaling Technology), α -Vinculin (V284, Bio-Rad). Antibodies used for flow cytometry: α -MR1 (26.5, conjugated to APC, Biolegend), α -HLA-A,B,C (W6/32, conjugated to APC, Biolegend), IgG2a isotype (MOPC-173, conjugated to APC, Biolegend), α -phospho-STAT1 (KIKSI0803, conjugated to PE, eBioscience).

Co-culture experiments

Primary AEC were infected with *S. pneumoniae* (20 MOI) in antibiotic-free BEGM. After 1 hour, AEC were washed with PBS to remove non-adhered bacteria, then MAIT cells were added at a 1:1 ratio in BEGM complete with gentamycin-amphotericin (GA-1000, Lonza). BEAS-2B cells in antibiotic-free DMEM-FBS were infected with *M. smegmatis* or treated with 6-FP or 5-OP-RU for 1 hour, washed with PBS, then MAIT cells were added at a 1:1 ratio in DMEM-FBS with gentamycin. Following overnight co-culture, wells were extensively washed with PBS to remove MAIT cells before harvesting AEC or BEAS-2B cells.

Real-time quantitative PCR

Cell pellets washed with PBS were either used fresh or stored dry at -80 °C before thawing in 37 °C water bath. RNA was isolated using the RNEasy Plus kit (Qiagen) and cDNA was synthesized using the High Capacity cDNA Reverse Transcription Kit (Life Technologies) as per the manufacturers' protocols. RT-qPCR was performed using TaqMan (Applied Biosystems) gene expression assays: *HPRT1* (Hs02800695_m1), *MR1* (Hs01042278_m1), *HLA-A,H* (Hs01058806_g1), *IRF1* (Hs00971965_m1), *NLRC5* (Hs01072123_m1), and β 2m (Hs00187842_m1). Gene expression data were normalized to internal control *HPRT1* and relative expression levels for each target gene were determined using the $2^{-\Delta\Delta C_t}$ method (99). Some uninfected AEC *HPRT1* and *MR1* data were used as controls in (22).

Flow cytometry

To quantify surface expression of MR1 and MHC-I, AEC and BEAS-2B cells were treated as indicated and harvested. Samples were blocked in FACS buffer containing 2% heat-inactivated human serum, 2% heat-inactivated goat serum, and 0.5% heat-inactivated FBS for 30 minutes on ice, then stained with APC-conjugated IgG2a, α -MR1, or α -HLA-A,B,C antibody for 40 minutes. For pSTAT1 staining, cells were permeabilized with 0.2% saponin during the blocking step. Cells were washed with PBS and fixed with 1% paraformaldehyde, then analyzed with a Beckman Coulter CytoflexS. All analyses were performed using FlowJo10 (TreeStar).

Enzyme-linked immunospot assays

IFN γ ELISPOT assays were performed as previously described (100) with the following modifications: ELISPOT plates (MSHAS4510, MilliporeSigma) were coated overnight with α -IFN γ antibody, then washed and blocked for 1 hour in RPMI-HuS. BEAS-2B cells were seeded in duplicate (1×10^5) cells/well and infected with *M. smegmatis*, treated with a titration of *M. smegmatis* supernatant, or incubated with control PHA or RPMI-HuS medium for 1 hour at 37 °C. D426G11 MAIT cell clones were added at a 1:1 ratio in RPMI-HuS with gentamycin for overnight incubation at 37 °C. Following extensive washing with PBS–0.05% Tween 20, plates were incubated with ALP secondary antibody for 2 hours before additional washing and colorimetric development. IFN γ spot-forming units (SFU) were quantified by AID ELISPOT reader. For experiments with cytokine pre-treatment, BEAS-2B cells were seeded in 6-well plates and treated with cytokines for 12 hours, then washed 3 times with PBS to remove any excess cytokine before harvesting and seeding into ELISPOT plate.

siRNA gene silencing

Gene silencing in wildtype, Cas9⁺, or NLRC5^{-/-} BEAS-2B cells was performed through nucleofection as in (101) and following the Amaxa Cell Line Nucleofector Kit T (Lonza) protocols. In brief, 2 μ g total of Missense (4390843, ThermoFisher), IRF1 (s7501, ThermoFisher), and/or NLRC5 (s38591, ThermoFisher) siRNA were added to 1×10^6 cells and transfected by the Amaxa Nucleofector 2b machine (Lonza) using program G-016. Cells were incubated for 48 hours before use in assays. Efficiency of gene silencing was validated by RT-qPCR.

Transcription factor binding sites

Putative transcription factor binding sites were acquired through the Eukaryotic Promoter Database browser using the Search Motif Tool to perform on-the-fly scanning for transcription factor motifs using the FindM tool from the Signal Search Analysis (SSA) Server toolkit (28, 102–104).

Data analysis

All data were analyzed using Prism (GraphPad) and plots were generated using R 4.4.0 and packages such as tidyverse, ggprism, and rstatix. Statistical significance was determined as indicated by two-tailed unpaired or pairwise t tests, using $\alpha=0.05$.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical approval was not required for the studies involving humans because materials used included 1) tissues from deceased human donors; 2) human serum from a biorepository (de-identified); 3) primary human T cell clone obtained as a gift (de-identified). The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent to participate in this study was not required from the participants or the participants' legal guardians/next of kin in accordance with the national legislation and the institutional requirements.

Author contributions

MEH: Writing – review & editing, Validation, Visualization, Data curation, Methodology, Formal analysis, Investigation, Conceptualization, Writing – original draft, Resources. EL: Methodology, Investigation, Data curation, Writing – review & editing, Resources, Project administration. TL: Investigation, Writing – review & editing, Data curation, Resources. CH: Data curation, Investigation, Resources, Writing – review & editing. MJH: Resources, Data curation, Formal analysis, Validation, Project administration, Writing – review & editing, Methodology, Funding acquisition, Supervision, Conceptualization, Writing – original draft.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2025.1624767/full#supplementary-material>

SUPPLEMENTARY FIGURE 1

Associated with **Figure 1** and **2**. Panels A–E are alternate presentations of data shown in **Figure 1A–E**, respectively. **(A)** RT-qPCR of RNA isolated from primary human AECs (n=5) infected with *S. pneumoniae* (Sp) for one hour and incubated overnight with MAIT cell clone. *MR1* expression was calculated relative to *HPRT1* expression and uninfected no-MAIT (UI-) controls, paired by individual donor. *MR1* **(B)** mRNA and **(C)** surface expression of BEAS-2B cells infected with *M. smegmatis* (Ms) for one hour and incubated overnight with MAIT cell clone. **(B)** RT-qPCR of *MR1* expression was calculated relative to *HPRT1* expression and UI- control, paired by experimental replicate. **(C)** Left, gMFI of surface *MR1* stained with α -MR1 26.5 Ab, paired by experimental replicate. Right, representative α -MR1 stain histograms. *MR1* **(D)** mRNA and **(E)** surface expression of BEAS-2B cells treated with 5-OP-RU (left, "5-OP") or 6-FP (right) for one hour and incubated overnight with MAIT cell clone. **(D)** RT-qPCR of *MR1* expression was calculated relative to *HPRT1* expression and media no-MAIT (UT-) control, paired by experimental replicate. **(E)** gMFI of surface *MR1* stained with α -26.5 Ab, paired by experimental replicate. **(F)** Representative α -MR1 stain histograms of BEAS-2B cells treated with media control (UT) or IFN γ . Data are representative of flow cytometry staining shown in **Figure 2D**. Pairwise statistical analyses are in **Supplementary Table 1**. Triangles represent data from primary AEC and circles represent data from BEAS-2B cells.

SUPPLEMENTARY FIGURE 2

Associated with **Figure 4**. **(A, B)** RT-qPCR of BEAS-2B cells treated with IRF1, NLRC5, and/or missense siRNA as indicated for 36 hours, then incubated with IFN γ for 12 hours. **(A)** *IRF1* and **(B)** *NLRC5* expression were calculated relative

to *HPRT1* expression and missense UT control, paired by experimental replicate. (C, D) RT-qPCR of Cas9⁺ or NLRC5^{-/-} BEAS-2B cells treated with IRF1 or missense siRNA for 36 hours, then incubated with IFN γ for 12 hours. Gene expression of (C) NLRC5^{-/-} clone #1 or (D) NLRC5^{-/-} clone #2 were calculated relative to *HPRT1* expression and Cas9⁺ or NLRC5^{-/-} clone missense UT controls, paired by experimental replicate. (E) Flow cytometry of cells from (C-D). gMFI of surface MR1 (α -26.5 Ab) are from IFN γ -treated Cas9⁺ (left), NLRC5^{-/-} clone #1 (middle), and NLRC5^{-/-} clone #2 (right). (F) RT-qPCR of Cas9⁺ or IRF1^{-/-} clone #1 BEAS-2B cells treated with IFN γ for 12 hours. *MR1* expression was calculated relative to *HPRT1* expression and Cas9⁺ or IRF1^{-/-} clone #1 UT controls, paired by experimental replicate. (G) Flow cytometry of Cas9⁺ or IRF1^{-/-} clone #2 BEAS-2B cells treated with IFN γ for 12 hours. gMFI of surface MR1 (left, α -26.5 Ab) and MHC-Ia (left, α -W6/32 Ab) are paired by experimental replicate. Statistical analyses are in **Supplementary Table 2**. Yellow symbols indicate IFN γ treatment alone. For visual clarity, silencing of IRF1 (green), NLRC5 (teal), or both (dark blue) are also indicated. In (F, G), light green distinguishes media control IRF1^{-/-} cells from IFN γ -treated IRF1^{-/-} cells (dark green).

SUPPLEMENTARY FIGURE 3

Associated with **Figure 5** and **Figure 6**. Panels (A–D, G) are alternate presentations of data shown in **Figure 5A, C, F, D, G**, respectively. Panels (E, F, H, I) are alternate presentations of data shown in **Figures 6A–D**,

respectively. (A) Left, flow cytometry of primary human AECs infected with *S. pneumoniae* (Sp) for one hour and incubated overnight with MAIT cell clone. gMFI of stained pSTAT1 expression is paired by individual donor (n=3). Right, histograms of α -pSTAT1 staining representative of data presented in **Figures 5A, B**. (B–D, G) RT-qPCR of primary human AECs infected with *S. pneumoniae* (Sp) for one hour and incubated overnight with MAIT cell clone. Expression of (B) $\beta 2m$, (C) *NLRC5* (D) *HLA-A*, and (G) *IRF1* were calculated relative to *HPRT1* expression and UI- control, paired by individual donor (n=4 (B) or n=5 (C, D, G) donors). (E, F, H, I) RT-qPCR of wildtype BEAS-2B cells treated as indicated below. Gene expression was calculated relative to *HPRT1* expression and UI- or UT- controls, paired by experiment. (E) *HLA-A* and (H) *IRF1* expression of BEAS-2B cells infected with *M. smegmatis* (Ms) for one hour and incubated overnight with MAIT cell clone. (F) *HLA-A* and (I) *IRF1* expression of BEAS-2B cells treated with 5-OP-RU (left) or 6-FP (right) for one hour and incubated overnight with MAIT cell clone. Pairwise statistical analyses are in **Supplementary Table 3**. Triangles represent data from primary AEC and circles represent data from BEAS-2B cells.

SUPPLEMENTARY FIGURE 4

Associated with **Figure 7**. (A–C) RT-qPCR of wildtype BEAS-2B cells treated with recombinant human cytokines for 12 hours. Gene expression was calculated relative to *HPRT1* expression and UT controls. Statistical analyses are in **Supplementary Table 5**.

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