Macrophage Coordination of the Interferon Lambda Immune Response

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Lambda interferons (IFN-λs) are a major component of the innate immune defense to viruses, bacteria, and fungi. In human liver, IFN-λ not only drives antiviral responses, but also promotes inflammation and fibrosis in viral and non-viral diseases. Here we demonstrate that macrophages are primary responders to IFN-λ, uniquely positioned to bridge the gap between IFN-λ producing cells and lymphocyte populations that are not intrinsically responsive to IFN-λ. While CD14+ monocytes do not express the IFN-λ receptor, IFNLR1, sensitivity is quickly gained upon differentiation to macrophages in vitro. IFN-λ stimulates macrophage cytotoxicity and phagocytosis as well as the secretion of pro-inflammatory cytokines and interferon stimulated genes that mediate immune cell chemotaxis and effector functions. In particular, IFN-λ induces CCR5 and CXCR3 chemokines, stimulating T and NK cell migration, as well as subsequent NK cell cytotoxicity. Using immunofluorescence and cell sorting techniques, we confirmed that human liver macrophages expressing CD14 and CD68 are highly responsive to IFN-λ ex vivo. Together, these data highlight a novel role for macrophages in shaping IFN-λ dependent immune responses both directly through pro-inflammatory activity and indirectly by recruiting and activating IFN-λ unresponsive lymphocytes.

Keywords: macrophage, interferon lambda, innate immunity, liver, Kupffer

INTRODUCTION

Lambda interferons (IFNL and IFN-λ), also known as type III IFNs, are a family of cytokines comprising four members: IFN-λ1 (IL29), IFN-λ2 (IL28A), IFN-λ3 (IL28B), and IFN-λ4. While all IFN-λs signal through a unique IFNLR1:IL10Rβ receptor complex, they activate a gene signature similar to type I IFNs, IFN-α, and IFN-β (1). Both type I and III IFNs activate the transcription of hundreds of interferon stimulated genes (ISGs) (1) that exhibit numerous autocrine and paracrine antiviral roles. Although IFNs are required to clear most viral infections, prolonged expression due to environmental or genetic factors can stimulate sustained immune activation, driving tissue damage, and fibrosis (2, 3).
Elevated IFN-λ3 production has demonstrated a strong association with IFNL genotype and hepatic inflammation, increasing the risk of both viral (HBV and HCV) and non-viral (non-alcoholic steatohepatitis, NASH) related progressive liver inflammation and fibrosis (4). Furthermore, these effects appear to be independent of IFN-λ4 activity, suggesting that IFN-λ3 may be a primary mediator of inflammation (5). While the precise mechanisms remain uncertain, peripheral and hepatic immune cell populations vary according to the IFNL polymorphism in patients with chronic HCV infection, suggesting that IFN-λ5s can prompt immune cell migration to tissues (5, 6).

IFN-λ activity is restricted to specific tissues due to selective IFNLR1 expression. In humans, epithelial cells within the lung, intestine and liver among others, are uniquely IFN-λ sensitive. In particular, IFN-λ s have been shown to protect against pulmonary influenza and human metapneumovirus (HMPV) (7, 8), gastrointestinal rotavirus and norovirus (9, 10) and hepatic HBV and HCV (11, 12). While the majority of human studies have been performed in vitro, murine studies have shown potent antiviral effects of IFN-λ s against numerous viruses including influenza and SARS coronavirus (13, 14), rotavirus, norovirus, and reovirus (15–17). It should be noted that IFNLR1 expression may differ between humans and mice, as exemplified in murine hepatocytes that do not respond to IFN-λ (18). Immune cells also demonstrate very restricted IFN-λ responsiveness with myeloid cell populations harboring the strongest responses: Human dendritic cells (DC) and neutrophils are highly responsive to IFN-λ s (19–23), whereas natural killer (NK) and T cells have consistently demonstrated minimal responsiveness (21, 24, 25). Investigation of monocyte and B cell responsiveness has produced conflicting results (21, 24, 26–30), perhaps confounded by studies utilizing co-culture models in the presence of IFN-λ responsive cells (31–33). As such, isolation of pure immune cell subsets is required to unequivocally define IFN-λ sensitivity.

Here, we demonstrate that human macrophages, not monocytes, are a dominant, physiologically relevant IFN-λ responsive population capable of orchestrating tissue inflammation. This is achieved through a direct immuno-stimulatory response to IFN-λ and subsequent NK and T cell chemotaxis and activation. In vivo, macrophages are responsive to IFN-λ3 and accumulate in inflamed human liver. These data suggest a novel role of macrophages as key players in modulating the IFN-λ response in acute infection, as well as chronic disease.

RESULTS

Macrophages Not Monocytes Are Responsive to IFN-λ

To address the uncertainty surrounding monocyte and macrophage (Mφ) IFN-λ responsiveness, we measured mRNA expression of the IFN-λ receptor, IFNLR1, in blood leukocytes by digital droplet PCR (ddPCR). ddPCR enables the precise quantification of RNA transcripts by performing the PCR reaction within >10,000 oil droplets, and calculating transcript copies using Poisson’s law of small numbers (34). IFNLRI expression in freshly isolated monocytes and in Mφs cultured for 7 days with GM-CSF was compared to IFN-λ responsive cells (pDCs) and “unresponsive” cells (NK and T cells). Similar to NK and T cells, monocytes expressed minimal IFNLRI transcript. Mφ and pDC IFNLRI expression was significantly increased compared to other populations, suggesting IFN-λ responsiveness (Figure 1A). Increased abundance of IFNLRI was confirmed following monocyte to macrophage differentiation using seven datasets from the NCBI Gene Expression Omnibus (35) (Figure S1). To assess IFNLRI expression during differentiation, IFNLRI expression was quantified over 24 h (qPCR, no differentiation stimulus) and 7 days (flow cytometry, GM-CSF differentiation) following monocyte plating. Expression of the IFNLRI transcript was quickly increased as early as 16 h post-plating, reaching a 30-fold increase at 24 h (Figure 1B). IFNLRI surface expression was significantly increased at day 3 (monocyte IFNLRI MFI 927 vs. day 3 Mφ 3199) and further increased at day 7 (day 7 Mφ IFNLRI MFI 10,412) (Figure 1C).

To test monocyte and Mφ responsiveness to IFN-λ, cells isolated and cultured as in Figure 1A were treated with 100 ng/ml IFN-λ3 for 8 h. This concentration is not a saturation dose, but is high enough to evoke a strong interferon response in Mφs (Figure S2). Consistent with increased IFNLRI expression, Mφs and pDC mRNA expression of viperin and ISG15 were markedly increased (Figure 1D), whereas monocytes and NK cells demonstrated negligible responses.

Differentiation Method Regulates IFN-λ Responsiveness

Mφ differentiation medium containing IFN-γ and LPS or interleukin 4 (IL-4) and IL-13 are often used to generate pro-inflammatory (M1) or anti-inflammatory (M2) Mφs, respectively, but do not reflect the spectrum of macrophage activation in vivo (36). To avoid generating Mφs whose IFN-λ sensitivity is influenced by phenotype skewing, monocytes were differentiated for 7 days with GM-CSF or M-CSF alone, as previously described (37, 38). The resulting Mφ populations are differentially responsive to inflammatory stimuli, and are thus M1- or M2-shifted while maintaining some baseline characteristics of polarized Mφs (Figure S3). When compared to monocyte derived DCs (MDDCs) generated using IL-4 and GM-CSF, the resulting Mφs express elevated surface expression of CD14 and CD16, reduced CD1C, and unlike MDDCs, adhere strongly to culture dishes (Figure S4). M1- and M2-shifted Mφs will be termed GM-Mφs and M-Mφs for the remainder of the manuscript.

IFN receptor expression and response to type I and III IFNs was examined in monocytes and Mφs. Mφ differentiation increases the abundance of the type I IFN receptor, IFNAR1 transcript (Figure 2A), and protein (Figure 2B) ~2-fold as compared to monocytes irrespective of stimulus. IFNLRI transcript abundance was increased in M-Mφs and GM-Mφs over 30- and 60-fold, respectively. The IFN-λ co-receptor IL10RB was also measured, and was not significantly modulated following macrophage differentiation (Figure S5). Consistent...
with gene expression, IFNLR1 protein was absent in monocytes, and increased in GM-Mφs compared to M-Mφs. The IFNLR1 bands at 70 and 45 kDa represent the full length and soluble isoforms of IFNLR1, respectively (24).

To confirm that elevated IFNLR1 expression confers response to IFN-λ, monocytes and Mφs from three healthy subjects were treated with IFN-λ3 for 15 min and STAT1 phosphorylation (Y701) was examined by Western blot. Monocytes did not phosphorylate STAT1 in response to IFN-λ3, whereas both M-Mφs and GM-Mφs were highly sensitive (Figure 2C). All cells demonstrated no STAT1 phosphorylation pre-treatment (Figure S6).

Mφs were subsequently treated with either interferon-alpha (IFN-α) or IFN-λ3 to determine whether cognate receptor expression defines sensitivity. Following 8h of IFN-α or IFN-λ3, all measured ISGs were significantly increased by both IFNs (Figure 2D). M-Mφs were more sensitive to IFN-α, demonstrating stronger induction of all ISGs, particularly CD80 and TRAIL. In contrast, GM-Mφs were more sensitive to IFN-λ3, increasing the expression of both ISG15 and viperin compared to M-Mφs. To confirm that macrophage differentiation and not treatment with M- or GM-CSF specifically induce IFNLR1 expression, monocytes were also differentiated using 10% autologous human serum from healthy individuals. Compared monocytes, human serum differentiated macrophages (HS-Mφs) possessed increased IFNLR1 transcript abundance and possessed similar IFN-λ3 responsiveness as MDDCs and GM-Mφs, both of which express high levels of IFNLR1 (Figure S7).

### IFN-λ3 Drives a Pro-inflammatory Macrophage Phenotype

The robust induction of IFNLR1 following monocyte plating suggests that monocytes quickly become IFN-λ- responsive upon differentiation and transmigration into tissues. Consequently, in the context of chronic antigen exposure, IFN-λ expression at sites of inflammation will likely influence monocyte differentiation and subsequent Mφ phenotype due to their prolonged exposure throughout the differentiation process. To test this hypothesis, we differentiated monocytes for 7 days with either M-CSF or GM-CSF alone (differentiation stimulus), or in combination with IFN-λ3 (activation stimulus), followed by transcriptional and functional assessment of Mφ phenotype (Figure 3A).
FIGURE 2 | M-CSF and GM-CSF differentiated macrophages respond differently to IFN-α and IFN-λ3. Following M- and GM-CSF stimulated differentiation, IFN-λ responsiveness of Mφ populations was examined, and compared to IFN-α. M-CSF and GM-CSF Mφ subsets both increased the expression of IFNAR1 ∼2-fold following differentiation from monocytes, and IFNLR1 transcripts ∼30- and 60-fold, respectively (A, n ≥ 9). Western blot of IFNAR1 and IFNLR1 from four healthy subjects confirmed these findings (B, n = 7, total). Supporting these findings, phosphorylation of STAT1 was detected by Western blot in both monocytes and macrophages following 15 min of IFN-α treatment and macrophages only following IFN-λ3 (C). ISG transcripts for ISG15, viperin, CD80, and TRAIL were examined to measure Mφ sensitivity to IFN-α (50 U/ml) and IFN-λ3 (100 ng/ml) (D, n = 8). M-CSF differentiated Mφs were more responsive to IFN-α, whereas GM-CSF differentiated Mφs, IFN-λ3. Data are representative of two independent experiments. Paired t-test *p < 0.05, **p < 0.01, ***p < 0.001 (mean ± SE). *Mock vs. IFN treatments, #M-Mφ vs. GM-Mφ.

RNA sequencing of M-Mφs and GM-Mφs from three donors was undertaken followed by paired analysis of transformed gene counts (Log 2) between untreated and IFN-λ3 treated Mφs (Tables S1, S2). The resulting smear plot demonstrates significantly up- and down-regulated genes following differentiation of M-Mφs (blue) and GM-Mφs (red) with IFN-λ3 (Figure 3B). GM-Mφs were significantly more responsive to IFN-λ3, up-regulating 463 genes ≥2-fold compared to 184 genes in M-Mφs. Similarly, GM-Mφs down-regulated 467 genes ≥2-fold compared to 252 genes in M-Mφs. IFN-λ driven ISG induction was also collectively higher in GM-Mφs as demonstrated by the heat map of gene expression LogFC (Figure 3C).

Functional analysis of data from Mφs differentiated with IFN-λ3 revealed numerous well defined ISGs (e.g., IFI27, MX1, and TLR3) and transcription factors (STAT and IRF gene families) responsible for ISG gene transcription (Figure 3D). In addition, a Th1 chemokine profile (CCL3, 4, and 5 and CXCL9, 10, and 11) responsible for CCR5 and CXCR3 mediated immune cell chemotaxis (39) was found in response to maturation with IFN-λ3, with stronger induction in GM-Mφs. Up-regulation of immune cell interaction and activation (CD80, CD86, and IL15) as well as antigen presentation [major histocompatibility complex (MHC) class I HLA genes] was also observed. Using transcriptomic markers of M1 and M2 Mφ differentiation, IFN-λ3 was found to induce the expression of the majority of M1, but not M2 markers, in both M and GM-differentiated Mφs, supporting the movement toward an M1 phenotype (GM-CSF p < 0.001, M-CSF p < 0.05, Sign test null hypothesis of 0.5) (Figure S8).

Gene induction was confirmed by qPCR from a larger group of donors including individuals used for RNA sequencing data, and compared to the effects of IFN-α differentiation. M-Mφs were considerably more sensitive to IFN-α, whereas
FIGURE 3 | Monocyte differentiation with IFN-λ3 drives a pro-inflammatory macrophage phenotype. Monocytes were cultured with M- or GM-CSF ± IFN-λ3 for 7 days to examine the effect of IFN-λ3 on Mφ differentiation, followed by phenotypic and functional characterization (A). RNA sequencing (n = 3/treatment) (Continued)
GM-Mφs responded strongly to IFN-λ (Figure 3E). In addition to chemokines identified by RNA sequencing, inflammatory mediators including CCL2, IL1B, and TNF transcripts were increased by IFN-λ in both Mφ subsets. To assess the role of differentiation (M- vs. GM-CSF), interferon treatment (IFN-α and -λ3), and their subsequent interaction, a 2-way ANOVA was additionally performed. As expected, all ISGs measured were significantly affected by IFN treatment (p < 0.01), however only CD80 expression was influenced by differentiation (p < 0.01). In agreement with RNA-Seq analysis, a significant interaction between IFN treatment and differentiation was observed for all measured genes (CXCL10, CCL8, IL15; p < 0.001, CD80; p < 0.01, TRAIL, TNF; p < 0.05) apart from IL1B and CCL2.

Analysis using Metacore functional annotation software demonstrated that similar networks were activated by IFN-λ3 in both M-Mφs and GM-Mφs (Table 1). Immune activation was considerably stronger in GM-Mφs, with highly significant p-values in networks such as antigen presentation and lymphocyte proliferation. Down-regulated gene networks were primarily associated with the cell cycle and protein translation (Table S3).

This analysis is consistent with Mφ BrdU assays, which demonstrated a reduction in BrdU incorporation following IFN-α (p < 0.05) and IFN-λ3 (NS) treatment (Figure S9).

**Interferon Lambda Promotes Lymphocyte Migration and NK Cell Degranulation**

To determine the extent at which Mφs differentiated with IFN-λ can drive immune cell migration, transwell migration assays were performed using autologous PBMCs. Following 24h transwell incubation, migrated cells were removed from the lower chamber and analyzed by flow cytometry (Figure 4A). IFN-λ3 stimulated lymphocyte migration solely in GM-Mφs by ~25% more than M-Mφs (Figure 4B), with NK, T, and NKT cell populations being most affected. Separate measurement of GM-Mφ media demonstrated that IFN-λ3 stimulated a significant increase in CCL2 (1.8×), CCL3 (3×), CCL4 (2.75×), and CXCL10 secretion (~5×), with minimal effect on M-Mφs (Figure 4C).

Co-culture experiments were next performed to assess the capacity of IFN-λ matured Mφs to stimulate NK cells in vitro. NK cells were incubated with Mφs overnight, removed, and co-cultured with K562 cells. K562 cells lack MHC class I expression, making them targets for NK cell killing. A significant increase in NK cell degranulation (CD107α), particularly within the CD56dim population, was observed following co-culture with IFN-λ3 treated GM-Mφs (Figure 4D). NK cell IFN-γ production was also increased following co-culture with IFN-λ3 treated GM-Mφs, but significance was lost within subgroup analysis. Minimal effect on NK cell function was observed following M-Mφ co-culture.

**IFN-λ Stimulates Macrophage Phagocytosis and Cytotoxicity**

To examine the effect of IFN-λ3 on Mφ effector function that is not associated with an inflammatory phenotype, phagocytosis was examined by flow cytometry. Tissue resident Mφs that demonstrate an M2 phenotype are highly phagocytic and efficient at presenting antigen (40), a phenotype that can be replicated in vitro (41, 42). UV induced apoptotic K562 cells stained with Zombie Yellow viability stain or DAPI labeled E. coli were incubated with Mφs for 1 h at a ratio of 2:1 and 4:1, respectively. The ratio of double-fluorescent (phagocytic, CD14+) cells to mono-fluorescent (non-phagocytic, CD14+) cells, as measured by flow cytometry (Figure 5A) was calculated to determine the phagocytic Mφ percentage (Figure 5B). Confocal microscopy was additionally used to confirm cell engulfment. IFN-λ3 increased phagocytosis of K562 cells (30% increase) and E. coli bacteria (10% increase) in M-Mφ alone. Mφ MFI, indicating the number of engulfed target cells, was consistent among populations when K562 cells were used as targets, likely reflecting

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**TABLE 1 | Metacore gene networks up-regulated by IFN-λ3.**

| M-CSF+IFN-λ3 Mφ networks | p-value |
|---------------------------|---------|
| Interferon signaling      | 9.25E-28 |
| Antigen presentation      | 2.69E-10 |
| Innate immune response to RNA viral infection | 4.08E-08 |
| Inflammasome              | 1.26E-07 |
| NK cell cytotoxicity      | 1.26E-06 |
| Chemotaxis                | 2.72E-06 |
| Lymphocyte proliferation  | 3.60E-06 |
| IL-10 anti-inflammatory response | 4.45E-06 |
| Phagosome in antigen presentation | 4.37E-05 |
| IFN-gamma signaling       | 4.57E-05 |

| GM-CSF+IFN-λ3 Mφ networks | p-value |
|---------------------------|---------|
| Interferon signaling      | 9.63E-22 |
| Antigen presentation      | 3.95E-17 |
| Lymphocyte proliferation  | 7.30E-14 |
| IL-4 signaling            | 7.17E-10 |
| Innate immune response to RNA viral infection | 6.34E-09 |
| NK cell cytotoxicity      | 1.59E-08 |
| Phagosome in antigen presentation | 6.91E-08 |
| Inflammasome              | 5.37E-07 |
| IFN-gamma signaling       | 2.07E-07 |
| Leucocyte chemotaxis      | 2.55E-07 |
Macrophages differentiated with IFN-λ3 induce lymphocyte chemotaxis and NK cell activation. Transwell inserts containing autologous PBMCs were placed into wells containing 6-day differentiated Mφs ± IFN-λ3 to examine immune cell chemotaxis (n = 6). Migrated cells were analyzed by flow cytometry using CD14, CD19, CD3, and CD56 antibodies to identify the number of migrated monocytes, B, NK, and T cells (A). The addition of IFN-λ3 to GM-CSF Mφs alone, stimulated lymphocyte migration, with significant increases in NK, NKT, and T cell migration (B). Consistent with migration data, GM-Mφ CCL2, CCL3, CCL4, and CXCL10 secretion were significantly increased by IFN-λ3, with no significant effect on M-Mφs (C) (n = 7). To assess the role of IFN-λ3 on Mφ activation of NK cells, autologous NK cells were incubated with Mφs for 16 h at a ratio of 1:1, removed and incubated with K562 target cells for a further 6 h to measure cytotoxicity by degranulation and IFN-γ expression (n = 4). GM-Mφs differentiated with IFN-λ3 significantly increased NK cell cytotoxicity as measured by CD107a expression, as well as IFN-γ production (D). Data are representative of two (B,D) and three (C) independent experiments. Paired t-test *p < 0.05, **p < 0.01 (mean ± SE). NE, No expression.

FIGURE 4 | Macrophages differentiated with IFN-λ3 induce lymphocyte chemotaxis and NK cell activation. Transwell inserts containing autologous PBMCs were placed into wells containing 6-day differentiated Mφs ± IFN-λ3 to examine immune cell chemotaxis (n = 6). Migrated cells were analyzed by flow cytometry using CD14, CD19, CD3, and CD56 antibodies to identify the number of migrated monocytes, B, NK, and T cells (A). The addition of IFN-λ3 to GM-CSF Mφs alone, stimulated lymphocyte migration, with significant increases in NK, NKT, and T cell migration (B). Consistent with migration data, GM-Mφ CCL2, CCL3, CCL4, and CXCL10 secretion were significantly increased by IFN-λ3, with no significant effect on M-Mφs (C) (n = 7). To assess the role of IFN-λ3 on Mφ activation of NK cells, autologous NK cells were incubated with Mφs for 16 h at a ratio of 1:1, removed and incubated with K562 target cells for a further 6 h to measure cytotoxicity by degranulation and IFN-γ expression (n = 4). GM-Mφs differentiated with IFN-λ3 significantly increased NK cell cytotoxicity as measured by CD107a expression, as well as IFN-γ production (D). Data are representative of two (B,D) and three (C) independent experiments. Paired t-test *p < 0.05, **p < 0.01 (mean ± SE). NE, No expression.
FIGURE 5 | IFN-λ3 stimulates macrophage phagocytosis of apoptotic cells. To examine the role of IFN-λ3 on macrophage phagocytosis, apoptotic K562 cells or E. coli were added to Mφ cultures for 1 h at a ratio of 2:1 and 4:1, respectively. Phagocytic Mφs were defined as cells double fluorescent for CD14 (BV711), and (Continued)
FIGURE 5 | Zombie/Yellow viability stain/DAPI, representing target cell engulfment (A). M-Mφs were more phagocytic than GM-Mφs toward K562 cells (2-fold increase, \( p < 0.05 \)), a phenotype that was further increased by IFN-\( \lambda \). Similarly, IFN-\( \lambda \) increased \( E_{\mathrm{cell}} \) engulfment in M-Mφs only. Mφ MFI, representing the number of cells engulfed remained unchanged in response to IFN-\( \lambda \). To assess cytotoxicity toward virus infected cells, Mφs were co-cultured with JFH1 infected Huh-7 cells, and apoptosis was quantified in Epcam\(^+\) Huh-7 cells using propidium iodide (PI) and Annexin V (C) \( (p = 7) \). IFN-\( \lambda \) stimulated GM-Mα cytotoxicity, increasing the percentage of early (Annexin V\(^+\)) and late (Annexin V\(^+\); PI\(^+\)) apoptotic cells, whereas early apoptosis alone was affected in M-Mφs (D). TRAIL expression was up-regulated by IFN-\( \lambda \) in GM-Mαs only, providing a possible mechanism of cytotoxicity (E). Data are representative of two independent experiments. Wilcoxon matched pairs signed rank test (B,E), paired \( t \)-test (D) \( p < 0.05, * p < 0.01 \) (mean ± SE).

their large size in comparison to Mφs. Conversely, Mφ DAPI MFI, representing bacterial engulfment, was significantly higher than M-Mφs, representing an increase in the number of phagocytosed bacteria. To determine the mechanism by which IFN-\( \lambda \) stimulates apoptosis, RNA-Seq data was queried, with a focus on Mφ receptors responsible for pathogen and apoptotic cell recognition. Consistent with an M2 phenotype, M-Mφs possessed higher expression of PRRs (TLR2, TLR4, and CD163), apoptotic ligand receptors (CD36 and MERTK) and complement transcripts (C1Q and C2) (Figure S10A). IFN-\( \lambda \) had minimal effect on the expression of most phagocytic receptors, but significantly increased key members of the complement cascade (C1S and C1R) (Figure S10B). These data suggest that activation of the complement system by IFN-\( \lambda \) may stimulate M-Mφs phagocytosis of both bacterial and apoptotic cells (43, 44), however further functional analysis is required.

To quantify the ability of Mφs to kill virus infected cells (cytotoxicity), Mφs were co-cultured with HCV infected (JFH1 strain) Huh-7 cells for 24 h. Following incubation, Huh-7 cells were labeled with Epcam, Annexin V, and propidium iodide (PI) to quantify cells undergoing apoptosis (Figure 5C). Additionally, Huh-7 and JFH1 infected Huh-7 cultures were used as controls to confirm HCV mediated Huh-7 cell apoptosis, as previously described (45). M- and GM-Mφs differentiated with IFN-\( \lambda \) stimulated an increase of early apoptotic (Annexin V\(^+\), PI\(^-\)) cells, by \( \sim 20 \) and \( 90\% \), respectively, compared to mock treated controls (Figure 5D). GM-Mφs alone increased Annexin V\(^+\), PI\(^+\) cells, representing late apoptosis by \( \sim 20\% \) following IFN-\( \lambda \) treatment. The cytoxic mechanism by which Mφs killed infected cells has not been determined, but is likely mediated by soluble factors such as TRAIL that is highly inducible following IFN-\( \lambda \) treatment in GM-Mφs in particular (Figure 5E). Low expression of TRAIL in untreated Mφs may explain the apparent lack of apoptosis following co-culture. In addition, no nitric oxide production by M- or GM-Mφs was found in response to IFN-\( \lambda \), bacterial or infected cell stimulus. To validate Huh-7 cell apoptosis results, qPCR for apoptosis markers Caspase 3 (Casp3), Caspase 7 (Casp7), and Bax were performed (Figure S11). Co-culture with IFN-\( \lambda \) differentiated GM-Mφs increased Casp3 and 7 expression by \( \sim 6\)-fold in addition to increasing the antiviral response of Huh-7 cells as demonstrated by strong induction of ISGs viperin and ISG15.

Liver Macrophages Are IFN-\( \lambda \) Responsive in vivo

To assess IFN-\( \lambda \) production in vivo, we measured the expression of IFNL genes in liver biopsies of chronic HBV, HCV, and NAFLD/NASH patients and compared them to normal liver tissue from benign liver resections. IFNLs were increased in both viral (>10-fold IFNL1, IFNL2/3 HCV vs. healthy) and non-viral (e.g., \( \sim 2\)-fold IFNL1, IFNL2/3 NAFLD/NASH vs. healthy) liver disease (Figure 6A), indicating that chronic inflammatory conditions can increase hepatic IFN-\( \lambda \) expression to facilitate the generation of inflammatory macrophages in vivo.

To demonstrate the presence of IFN-\( \lambda \) responsive Mφs in vivo, we performed immunofluorescent labeling of liver tissue from a patient with autoimmune hepatitis (AIH), chronic HCV infection, and normal liver obtained from a cancer resection. Biopsies were labeled with IFNLR1 and CD68 or CD11b antibodies to identify IFN-\( \lambda \) responsive liver Mφs (Kupffer cells) or myeloid populations (monocytes/macrophages/neutrophils), respectively. As demonstrated in Figure 6B, all CD68\(^+\) and a fraction of CD11b\(^+\) cells were labeled with IFNLR1.

Immunolabeling was also performed using CD68 or CD11b in combination with CD3 to demonstrate immune cell proximity in inflamed tissue (Figure S12). CD3\(^+\) T cells localized in proximity to CD68 Mφs, supporting a role for Mφ derived chemokines as mediators of immune cell trafficking.

To confirm that liver Mφs are responsive to IFN-\( \lambda \), CD68\(^+\) Mφs were harvested from liver resection tissue by cell sorting (Figure 6C), and treated with 100 ng/ml IFNL-\( \lambda \) ex vivo. Consistent with our in vitro findings, liver Mφs highly express IFNLR1 compared to liver NK (CD3\(^-\) and CD56\(^+\)), NKT (CD3\(^+\) and CD56\(^-\)), and T cells (CD3\(^+\) and CD56\(^-\)) (Figure 6D). In addition, Mφ IFNLR1 MFI negatively correlated with blood white blood cell count \( (r = -0.678, p < 0.05) \) and positively correlated with hepatic T cell enrichment as a percentage of CD45 cells \( (r = 0.712, p < 0.05) \).

To compare IFN-\( \lambda \) sensitivity, T cells and Mφs from each individual were cultured for 10 h in media alone or with IFN-\( \lambda \), followed by quantification of ISG expression. T cells were unresponsive to IFN-\( \lambda \) as demonstrated by a lack of ISG15 and viperin induction (Figure 6E). Conversely, IFNLR1 expressing Mφs were highly responsive to IFN-\( \lambda \), increasing the abundance of both transcripts \( \sim 6\)-fold.

DISCUSSION

The cellular and molecular mechanisms by which IFN-\( \lambda \) modulates host responses to viral infections and tissue inflammation remains unclear. Here we undertook comprehensive functional characterization to demonstrate both in vitro and ex vivo, that macrophages are likely immune cell drivers of IFN-\( \lambda \) mediated hepatic antiviral and inflammatory activities. Unlike monocytes, macrophages are highly sensitive to IFN-\( \lambda \) through the induction of IFNLR1
FIGURE 6 | Hepatic IFN-λ3 responsive macrophages are present in vivo. IFNL1, IFNL2/3, and IFNLR1 mRNA expression was measured in healthy, NAFLD/NASH, HBV, and HCV infected liver tissue (n ≥ 9/group) (A). To examine IFNLR1 expression in vivo, biopsy sections obtained from healthy, autoimmune hepatitis, and HCV (Continued)
expression. As such, monocytes likely become IFN-λ responsive upon movement into tissue and subsequent differentiation. Upon IFN-λ stimulation, macrophages develop a robust immune-stimulatory gene signature, expressing hundreds of ISGs, cytokines, chemokines, and co-stimulatory molecules to stimulate both autocrine and paracrine immune cell activation (Figure 7).

IFN-λs are inducible cytokines that drastically increase in abundance upon viral infections, but can also effectivly protect against bacterial and fungal insults (46, 47). Activation of TLRs 3, 4, 5, 7, 9 (48, 49) can drive IFN-λ expression, which is dependent factors including cell type and cellular environment. IFN-λs are necessary for epithelial barrier protection in the lungs, liver and gastrointestinal tract, however their dysregulation has been associated with a number of diseases that lack an obvious association with microbial infection. These include chronic inflammatory diseases such as psoriasis (50), systemic lupus erythematosus (51), and asthma (52). Consequently, it is important to understand the direct and indirect molecular mechanisms by which IFN-λs are induced, as well as the responding cellular identities.
The effectiveness of direct acting antiviral therapy for chronic HCV infection has ultimately overshadowed the antiviral role of IFN-λs in the liver, however there remains much to be understood regarding the immuno-stimulatory and potentially destructive roles of these unique cytokines. Recent evidence suggests that IFNL genotype influences IFN-λ expression in the liver to facilitate immune cell migration and subsequent inflammation (4, 5). Unlike IFN-λ4 which is weakly secreted by hepatocytes (53, 54), IFN-λ1–3 are highly expressed, and can exert paracrine effects on surrounding immune cells. This is consistent with reports showing increased Mφ activation in patients possessing the favorable IFNL genotype (55). The importance of the Mφ response is additionally underscored by the fact that Mφ but not hepatocyte ISG expression is positively associated with both the favorable IFNL genotype that produces increased IFN-λ3 and antiviral response (56, 57).

Both PCR and RNA-Seq analysis support the IFN-λ sensitive nature of GM-Mφs and highlight the stimulatory role of IFN-λ3. Increased expression of pattern recognition receptors [TLR3, IFH1 [MDA5], DDX58 [RIG-1]] in response to IFN-λ can increase antigen recognition, as we have shown in Figure 5. Numerous pro-inflammatory transcription factors including STATs 1–3, IRFs 1, 7, and 9, AP-1, and NFκB components were activated in response to IFN-λ, as demonstrated by an enrichment of their respective target genes (Table S4). Inflammatory cytokines TNF and IL1B that are known mediators of hepatocyte apoptosis and liver injury were moderately, albeit significantly induced by IFN-λ treatment alone (Figure 3E), though our data supports additional inflammatory effects of IFN-λ. By strengthening Mφ recognition and response to pathogen associated molecular patterns, IFN-λ.s likely sensitize Mφs to inflammatory stimuli, thus amplifying the strength and/or duration of the inflammatory cascade. This has been demonstrated by Liu et al. who showed that IFN-λ.1 can promote IL-12 production in TLR7 stimulated Mφs (28).

In response to IFN-λ, GM-Mφs potently express Th1 chemokines including CXCL 9, 10, and 11 as well as IL-15 and IL-27, notable drivers of T and NK cell activation and proliferation. In agreement with RNA-Seq gene expression data, we demonstrated that IFN-λ3 treated GM-Mφs stimulate T and NK cell chemotaxis and subsequent NK cell cytotoxicity. These data suggest that IFN-λ.s are strong mediators of the Th1 response and provides a rationale for works by Morrow et al. who showed that IFN-λ3 can increase IFN-γ secretion and degranulation despite T cell insensitivity to IFN-λ. (58). A similar phenotype has been observed in tumor model NK cells, where IFN-λ signaling drives NK cell cytotoxicity, suppression of tumor growth and metastases (33, 59). This Th1 skewing effect has been further validated using murine models of Th2 diseases where IFN-λ alleviated symptoms of airway disease (60), intestinal inflammation (61), and conjunctivitis (62).

Interestingly, GM-Mφs were significantly more responsive to IFN-λ, whereas M-Mφs more responsive to IFN-α. These data suggest that while type I and type III IFNs induce a similar gene signature, their respective response is dependent not only on the cell type, but also the, the inflammatory phenotype of the responsive cell. This data is consistent with work by Fleetwood et al. that demonstrate a strong dependence on type I IFN signaling in M-CSF over GM-CSF cultured Mφs (37). Consistent with an M2 phenotype (63), M-Mφs were not particularly efficient at driving immune cell chemotaxis and activation upon IFN-λ3 stimulation, but were significantly more phagocytic than GM-Mφs both at baseline and in response to IFN-λ3. These data suggest that IFN-λ.s are perhaps not inherently inflammatory, but instead promote macrophage effector functions based on location or developmental phenotype.

Our data fills a current gap in knowledge concerning the cellular identities and mechanisms that regulate local IFN-λ mediated inflammation. Because IFN-λ signaling is longer lasting and unlike IFN-α does not become refractory following chronic exposure (64), continuous IFN-λ expression from chronic infections can drive prolonged immune activation. While DCs have a strong IFN-λ response, they are a small population in the liver and migrate toward proximal lymph nodes in response to infection (65). Liver Mφs (Kupffer cells) on the other hand consist of the ~3/4 of hepatic immune cells, and remain locally to become crucial drivers of localized tissue inflammation (65). Neutrophils are the only other immune cell subset with a defined IFN-λ response, and respond with reduced migration and suppression of inflammation (22, 23, 66).

In summary, we have demonstrated a novel concept whereby Mφs gain IFN-λ sensitivity quickly following differentiation from monocytes. These data support a pro-inflammatory role for IFN-λ.s, particularly via recruitment of NK and T cells, chief promoters of inflammation in chronic liver disease. Mφs bridge the gap between IFN-λ responsive and non-responsive effector cells, and are likely implicated in the elimination of acute infection and the promotion of tissue damage in chronic disease.

METHODS
Patient Cohort
Blood samples were obtained from healthy volunteers at the Westmead Institute of Medical Research. Data points represent individual donors from a cohort of ~20 healthy individuals. Different cohorts of donors were used for individual experiments based on availability. Liver tissue was obtained at Westmead Hospital, Sydney, at the time of needle biopsy [chronic HBV/HCV infection, non-alcoholic fatty liver disease (NAFLD)/NASH, autoimmune hepatitis] or from patients undergoing liver resections (normal tissue). Ethics approval was obtained from the Sydney West Area Health Service and University of Sydney. Informed consent was obtained for all subjects [HREC2002/12/4.9(1564)].

Immune Cell Isolation, Culture, and IFN Treatment
Peripheral blood mononuclear cells (PBMCs) were isolated from volunteer blood using Ficoll Paque Plus (GE Healthcare). Immune cell isolations were performed using StemCell EasySep Kits, resulting in immune cell purity of >90%. CD14+ monocytes were cultured at 37°C and 5% CO2 in RPMI medium containing 10% fetal calf serum (FCS) and 50 ng/ml macrophage colony-stimulating factor (M-CSF, Peprotech) or granulocyte...
macrophage colony-stimulating factor (GM-CSF, Peprotech) for 7 days, replacing media and removing non-adherent cells at day 4. Mφ populations were treated with IFN-α (50 U/ml) or IFN-λ3 (100 ng/ml) purchased from R&D Systems. All cytokines were confirmed LPS free.

**RNA Sequencing and Bioinformatics**

RNA was extracted using the Favorgen Tissue Total RNA Kit and the sequencing library was prepared using the TrueSeq Stranded mRNA Library Prep Kit (Illumina). Single ended RNA sequencing (RNA-Seq) was performed at the Australian Genome Research Facility using the Illumina HiSeq 2500 platform (50 bp read length; minimum of 10^7 reads per sample). Sequence alignments and gene counts were performed using STAR RNA-Seq aligner version 2.5.1b (67) and paired comparisons were performed using EdgeR version 3.16.2(68). Heat map visualization of RNA-Seq data was performed using GITools (69). Functional analysis of IFN-λ3 mediated gene expression was conducted using Metacore version 6.29 (Thomson Reuters).

**Quantitative PCR**

cDNA synthesis was performed using MMLV reverse transcriptase (Promega) and 500 ng of RNA. Gene transcripts were quantified using the Corbett Research Rotorgene 3000 or 6000 thermocyclers with TaqMan primer probes (Applied Biosystems) or custom primers. Quantification of CD80, CXCL10, IFNLR1, and TRAIL were performed using primer probes (Applied Biosystems). Custom primers sets are as follows: CCL2 (CTGCTCATAGAGCCACCTT, GCACTGAGATCTTTCCATTTG), CCL8 (TCCCAAGGAAGCTTGATCTT, ATGGAATCCCTGACCATC), IFNAR1 (TCAGGTGTAGAAGAAAGGATG, AGACACAAATTTGCCATGCA), IFNL1 (AGGGAGCCTTTGAAGT, AGAGT, GAAGCCTCAGGTCCCAATTC), IFNL2/3 (GCCCACTGCTTCAAGTC, GGGCATCTGGCCCTAATA), IL1B (TCGCCAGTGAATAGGCT, GGTGGAGATTGG), IL15 (CTGAGTTACCCCATGTG, CATCTCCGACTCACTG), IFNAR1 (TCAGGTGTAGAAGAAAGGATG, AGACACAAATTTGCCATGCA), IFNL1 (AGGGAGCCTTTGAAGT, AGAGT, GAAGCCTCAGGTCCCAATTC), IFNL2/3 (GCCCACTGCTTCAAGTC, GGGCATCTGGCCCTAATA), and viperin (CCTTTGCTGAGGCTCTTTAG, CAGCTGCTGTTTCTGCTT). All transcripts were normalized to 18s ribosomal RNA (Applied Biosystems, 4319413E). Standard curves derived from combined assay RNA were used to determine relative expression of genes.

**Digital Droplet PCR (ddPCR)**

Immune cell RNA was quantified using the Qubit fluorometer and RNA BR assay kit (Thermo Fisher), and cDNA was synthesized from ≥10 ng of RNA per sample using qScript cDNA supermix (Quantabio). cDNA was combined with ddPCR supermix and droplet generation oil for probes (Bio-Rad), and droplets were generated using the Bio-Rad QX200 Droplet Generator. PCR was performed using IFNL1R and GAPDH probes according to the manufacturer's instructions, and droplet fluorescence was analyzed using the Bio-Rad QX200 Droplet Reader. Absolute quantification of transcript number was determined using QuantaSoft Analysis Pro software.

**Western Blotting**

Mφs were lysed at 4°C using a denaturing buffer containing protease and phosphatase inhibitors. Protein was quantified and subject to sodium dodecyl sulfate poly-acrylamide gel electrophoresis. Gels were transferred to nitrocellulose membranes, blocked and probed with: IFNAR1 (Abcam, ab45172), IFNL1R1 (Sigma Aldrich, HPA017319), STAT1 (Santa Cruz Biotechnology, SC-345), p-STAT1 (Cell Signaling, 9167), and β-actin (Sigma-Aldrich, A1978). Protein bands were visualized on X-ray film using horseradish peroxidase (HRP) conjugated secondary antibodies and the Supersignal West Pico chemiluminescence kit (Pierce Endogen).

**Chemotaxis Assays and ELISAs**

Immune cell chemotaxis assays were performed using 1 × 10^6 autologous PBMCs placed in 5 μM pore size transwell inserts. Assays were performed for 24 h at 37°C and 5% CO2. Migrated cells present in the lower chamber were removed by pipetting and characterized by flow cytometry. Zombie Aqua viability stain (BioLegend) and antibodies directed toward CD19 (BioLegend, 302218), CD3 (BioLegend, 300424), CD56 (Becton Dickinson, 335791), and CD14 (Becton Dickinson, 563372) were used to identify immune cell populations. All samples examined by flow cytometry have been treated with Fc block (BD, 564219) prior to staining. ELISAs for CCL2 (R&D Systems, DY279), CCL3 (R&D Systems, DY270), CCL4 (R&D Systems, DY271), CXCL10 (BioLegend, 439904), and TRAIL (Abcam, ab46074) were performed according to the manufacturers’ protocols.

**Phagocytosis Assays**

To stimulate apoptosis, K562 cells were exposed to UV light for 15 min. Apoptotic K562 cells were stained with ZombieYellow viability stain (Biolegend), after which apoptosis was confirmed with >90% of cells staining positive. Culture media was removed from Mφ cultures and target cells in RPMI + 10% FCS were added at a ratio of 2:1 (K562). Culture plates were centrifuged at 450 g for 2 min to synchronize phagocytosis. Following 1 h of incubation at 37°C, cells were washed and labeled with BV711 mouse anti-human CD14 antibody (BD Biosciences), and analyzed using the BD Biosciences LSR Fortessa cell analyzer.

**Cytotoxicity Assay**

Huh-7 cells were electroporated with JFH1 RNA, a genotype 2 strain of HCV as previously described (70). Upon confirming >85% infection rate by HCV NS5A immunofluorescence, Huh-7 cells were plated in 48 well plates. Day 6 Mφs were spun down onto Huh-7 cells at 400 × g for 5 min at a ratio of 2:1. Following 24 h incubation, macrophages were removed by pipetting, and Huh-7 cells detached using Accutase (Sigma-Aldrich). Huh-7 cells were labeled with Annexin V (Becton Dickinson, 550474), Epcam (Miltenyi Biotec, 130-091253) and propidium iodide (Sigma Aldrich, P4864) in 1×
Annexin V binding buffer according to the manufacturers protocol. Cells were washed thoroughly, fixed with 2% paraformaldehyde and analyzed on the BD Biosciences LSR II cell analyzer.

**NK Cell Degranulation and Interferon Gamma Production**

Following 6 days of culture, freshly isolated autologous NK cells were added to Mφs at a ratio of 1:1. Cells were centrifuged at 300 g for 3 min and incubated for 16 h at 37°C with 5% CO2. Following stimulation by Mφs, NK cells were removed by pipetting, and incubated ± K562 cells (1:1 ratio) with an antibody against the degranulation marker CD107a (BD Biosciences 328620), GolgiStop and GolgiPlug transport inhibitor for 6 h at 37°C with 5% CO2. NK cell degranulation and interferon gamma (BioLegend, 502509) production was examined by flow cytometry, identifying live NK cell populations using Zombie Aqua viability stain (BioLegend 423101), APC-CY7 CD19 (BioLegend, 302218), Alexafluor 700 CD3 (BioLegend, 300424), PE-CY7 CD56 (Becton Dickinson, 335791), and BUV395 CD16 (Becton Dickinson, 563785).

**Immunofluorescence**

Frozen biopsy tissue sections were fixed with acetone, blocked and labeled with primary antibodies against CD11b (Bio-Rad MCA74AA488), CD68 (Abcam, AB955), and IFNLR1 (all 1:100 dilution) overnight at 4°C. Secondary fluorescent antibodies (Alexafluor 488/594 anti-rabbit/mouse, Life Technologies, 1:1,000 dilution) and DAPI were applied for 1 h at room temperature, and imaged by confocal microscopy (Olympus FluoView FV1000). The IFNLR1 antibody was validated using siRNA knockdown of IFNLR1 on macrophage cultures, as well as gastrointestinal biopsy tissue to ensure specific labeling (Figure S13).

**Immune Cell Sorting**

Liver tissue was diced and incubated for 30 min at 37°C in a dissociation buffer consisting of RPMI medium containing 1 μg/ml DNase, 0.1 mg/ml Collagenase type IV, and 100 U/ml penicillin/streptomycin. Cells were filtered through a 70 μm cell strainer and centrifuged at 50 g for 5 min to pellet hepatocytes (71). The supernatant containing liver immune cells was washed, pelleted at 400 g and frozen at −80°C until a sufficient number of samples were obtained. Fluorescence-activated cell sorting (FACS) was performed using the Becton Dickinson Influx Cell Sorter using the following panel: Zombie Aqua viability stain (BioLegend, 423102), APC CD45 (BioLegend, 304012), BUV395 CD3 (Becton Dickinson, 563546), PE-CY7 CD56 (Becton Dickinson, 335791), BV711 CD14 (Becton Dickinson, 563372), Alexafluor 488 CD68 (BioLegend, 333812), and PE IFNLR1 (BioLegend, 337804). A cell sort purity of >90% was measured during sorting.

**Statistics**

Statistics were performed using GraphPad Prism and were chosen based on the normality of the data, with p < 0.05 deemed significant. Student t-tests or Mann–Whitney tests were performed on unpaired samples based on data normality. Paired t-tests and Wilcoxon matched pairs signed rank test were performed on paired samples based on Gaussian distribution.

**DATA AVAILABILITY STATEMENT**

RNA sequencing data has been uploaded into the Figshare data repository: https://doi.org/10.6084/m9.figshare.10324511.v1.

**ETHICS STATEMENT**

The studies involving human participants were reviewed and approved by Sydney West Area Health Service. The patients/participants provided their written informed consent to participate in this study.

**AUTHOR CONTRIBUTIONS**

SR, ET, MR-M, SS, DB, and GA: designing research studies. SR, RW, ET, and MR-M: conducting experiments. SR, RW, CL, and GA: acquiring and analyzing the data. VL, LY, DB, MD, JG, and GA: providing tissues and reagents and interpreting results. SR, MD, JG, and GA: writing the manuscript.

**FUNDING**

This project was supported by the Robert W. Storr Bequest to the Sydney Medical Foundation, University of Sydney; a National Health and Medical Research Council of Australia (NHMRC) Program Grant No. 1053206. GA was supported by a Sylvia and Charles Viertel Charitable Foundation Investigatorship (VTL2015C022).

**ACKNOWLEDGMENTS**

RNA sequencing, flow cytometry and microscopy were performed in the Genomics, Flow Cytometry and Cell Imaging Core Facilities that are supported by the Westmead Research Hub, Cancer Institute New South Wales and National Health and Medical Research Council. In particular, we would like to thank Suat Dervish and Edwin Lau for their expertise and help performing cell sorting experiments and Joey Lai for his help preparing mRNA libraries for RNA sequencing.

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2019.02674/full#supplementary-material
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