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Differential production of type I IFN determines the reciprocal levels of IL-10 and proinflammatory cytokines produced by C57BL/6 and BALB/c macrophages

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Abstract

Pattern recognition receptors (PRR) detect microbial products and induce cytokines, which shape the immunological response. Interleukin-12 (IL-12), tumor necrosis factor alpha (TNF-α) and IL-1β are proinflammatory cytokines, which are essential for resistance against infection, but if produced at high levels, may contribute to immunopathology. In contrast, IL-10 is an immunosuppressive cytokine, which dampens proinflammatory responses, but can also lead to defective pathogen clearance. The regulation of these cytokines is therefore central to the generation of an effective but balanced immune response. Here, we show that macrophages derived from C57BL/6 mice produce low levels of IL-12, TNF-α and IL-1β, but high levels of IL-10 in response to TLR4 and TLR2 ligands LPS and PamCSK4, and *Burkholderia pseudomallei* a Gram-negative bacterium which activates TLR 2/4. In contrast, macrophages derived from BALB/c mice show a reciprocal pattern of cytokine production. Differential production of IL-10 in *B. pseudomallei* and LPS stimulated C57BL/6 and BALB/c macrophages was due to a type I IFN and ERK1/2-dependent, but IL-27 independent mechanism. Enhanced type I IFN expression in

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Disclosures

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LPS-stimulated C57BL/6 macrophages was accompanied by increased STAT1 and IRF3 activation. Further, type I IFN contributed to differential IL-1β and IL-12 production in *B. pseudomallei* and LPS stimulated C57BL/6 and BALB/c macrophages, via both IL-10-dependent and independent mechanisms. These findings highlight key pathways responsible for the regulation of pro- and anti-inflammatory cytokines in macrophages and reveal how they may differ according to the genetic background of the host.

Introduction

Proinflammatory immune responses are critical in the defence against pathogens, however, excessive inflammation has the potential to cause damage to the host. Thus, immunoregulatory pathways controlling inflammatory cytokine production are critical in ensuring an effective but balanced immune response (1). Pattern recognition receptor (PRR) activated macrophages are an important early source of proinflammatory cytokines such as interleukin (IL)-12, tumor necrosis factor (TNF)-α and IL-1β, and their production is modulated by a complex array of direct and indirect regulatory mechanisms (1, 2).

A central negative regulator of inflammatory responses is the immunosuppressive cytokine IL-10 (3). The spontaneous onset of colitis in response to commensal gut flora in IL-10 deficient mice (4) and enhanced susceptibility of IL-10 deficient mice to septic shock (5), demonstrate the importance of IL-10 as negative feedback regulator in the immune system. IL-10 can be produced by several cell types within the immune system, including PRR-stimulated macrophages (6), to which IL-10 can signal back in an autocrine manner and inhibit the production of proinflammatory cytokines via a STAT3 dependent mechanism (7–9).

Type I interferons (IFN), constitute a group of cytokines including IFN-β and multiple IFN-α proteins (10). Studies of the functions of type I IFN have revealed complex immunoregulatory roles for these cytokines. For example, type I IFN has been shown to promote the production of IL-10 from murine macrophages (11), human monocytes (12) and human DCs (13), although the mechanisms by which this occurs are not fully understood. In the context of proinflammatory cytokine production, the effects of type I IFN are diverse. Mechanisms whereby type I IFN regulates distinct proinflammatory cytokines are as yet unclear (14–19). The role of type I IFN *in vivo* in the context of infection and inflammation is complex (20). Type I IFN has been shown in different settings to contribute to control of the pathogen or conversely may regulate or exacerbate inflammatory pathologies (20).

C57BL/6 and BALB/c mice differ significantly in their immune responses giving rise to distinct outcomes of infection and have provided robust models for studying susceptibility or resistance to various pathogens (21–23). *Burkholderia pseudomallei*, is a Gram-negative bacterium and the causative agent of melioidosis, a major cause of sepsis and mortality in endemic regions of South East Asia and Northern Australia which is being increasingly being reported across the tropics. There is no available vaccine, antibiotic treatment is prolonged and not always effective and mortality rates in acute cases can approach 50% even with optimal clinical management (24). Both proinflammatory cytokines and IL-10 are found at high levels in the plasma of individuals with acute infection and their

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concentrations can predict mortality (25). Infection with *B. pseudomallei* serves as an important clinical and experimental example of Gram-negative sepsis and resistance to infection is genetically determined. Several studies have shown that BALB/c mice, even when infected at low dose, will develop acute disease and succumb much earlier than C57BL/6 mice, which conversely can establish a longer-term chronic infection with this pathogen if infected with a low bacterial load (26–28). Relative susceptibility to *B. pseudomallei* infection has been correlated with distinct profiles of proinflammatory cytokines produced by innate cells in addition to IFN-γ, in *B. pseudomallei* infected C57BL/6 and BALB/c mice (28–31). However, the complexities of *in vivo* infection models have made it difficult to fully dissect the mechanisms underlying differential cytokine production between these two strains of mice. Similarly, in the context of colitis, a disease associated with the elevated production of several proinflammatory cytokines, IL-10 deficient BALB/c mice are more susceptible than IL-10 deficient C57BL/6 mice (32), but again the mechanisms underlying this phenotype are incompletely understood. Thus, the *in vitro* study of cellular immune responses from these mice provides a valuable comparative model for the mechanistic dissection of cytokine regulation, which additionally may contribute to differences in resistance of C57BL/6 and BALB/c mice to infection and inflammatory diseases.

We report here reciprocal profiles of IL-10 versus IL-12, TNF-α and IL-1β production from C57BL/6 and BALB/c macrophages stimulated with *B. pseudomallei* and purified TLR2 and TLR4 ligands. Our investigation into these phenotypes revealed type I IFN to be a central mediator of differential cytokine production in C57BL/6 and BALB/c macrophages. Enhanced type I IFN production accounted for the reduced levels of IL-1β and IL-12 observed in C57BL/6 as compared to BALB/c macrophages, by IL-10-dependent and independent mechanisms. We also show that prolonged IL-10 expression in C57BL/6 macrophages results from type I IFN-induced ERK1/2 activation. These results support an important immunoregulatory role for type I IFN together with IL-10 and demonstrate that this activity is dependent upon the genetic background of the host.

Materials and methods

Animals

C57BL/6 Wild Type (WT), BALB/c WT, C57BL/6 *Il10*$^{-/-}$, BALB/c *Il10*$^{-/-}$ and all other mutant mice were bred and maintained at The Francis Crick Institute, Mill Hill Laboratory under specific pathogen-free conditions in accordance with the Home Office, UK, Animal Scientific Procedures Act, 1986. *Tlr4*$^{-/-}$ and *Trif*$^{-/-}$ breeding pairs, all on a C57BL/6 background, were provided by Professor S. Akira (Osaka University, Osaka, Japan). C57BL/6 *Ifnar1*$^{-/-}$ breeders originated from B&K Universal (Hull), UK, and C57BL/6 *Tccr*$^{-/-}$ (referred to as *Il27ra*$^{-/-}$ in text) breeders were provided by Genentech, CA, USA. All mice used were female, between 8-16 wk of age.

Generation and stimulation of bone marrow derived macrophages (BMDMs)

BMDMs were generated as previously described (33). On day 6, adherent cells were harvested and seeded in 48-well tissue culture plates at 0.5×10^6 cells/well and rested for

18-20 h prior to stimulation. Cells were stimulated, unless otherwise stated, with 10 ng/ml *Salmonella minnesota* LPS (Alexis), 200 ng/ml Pam3CSK4 (InvivoGen, Fr) or heat-killed (to avoid heavy Containment Level 3 work) *B. pseudomallei* 576 (Bps) at a ratio of 5-500 *B. pseudomallei* to 1 BMDM. Data were verified to be similar at the cytokine protein and mRNA level using live *B. pseudomallei* 576 (Bps) (data not shown). When indicated, cells were treated with recombinant IFN-β (PBL) or recombinant IL-27 (R&D), both of which were shown to have very low levels of < 1 EU/μg endotoxin only, which on dilution in LPS free media for assay resulted in < 0.02 EU/ml. Antibodies, 10 μg/ml anti-IFNAR1 mAb (clone MAR1-5A3, mouse IgG1, Bio X Cell), 10 μg/ml anti-IL-10R (clone 1B1.3a, rat IgG1) or relevant isotype control (clones GL113 or TC31.2F11, respectively), were all gifts from DNAX (now Merck, Palo Alto, CA, USA). MEK inhibitor PD184352 (1 μM) or PD0325901 (0.1 μM) and p38 inhibitor SB203580 (0.5 μM) were added to BMDMs 1 h prior or 2 h post stimulation with LPS as indicated (34).

Cytokine quantification

Cytokines were quantified from supernatants of stimulated cells. IL-10 and IL-12p40 were quantified by ELISA. Cone JES5-2A5 (eBiosciences) was used for IL-10 capture and biotinylated anti-mouse IL-10 SXC-1 (BD Biosciences) for detection. Clone C15.6.7 was used for IL-12p40 capture and biotinylated anti-mouse IL-12p40 C17.8 for detection (both gifts from DNAX), followed by HRP conjugated Streptavidin (Jackson ImmunoResearch Laboratories Inc.). IL-12p70, TNF-α and IL-27 (eBioscience), IL-1β (R&D) and IFN-β (PBL) were quantified using commercially available ELISA kits.

RNA isolation and quantitative real-time PCR (qRT-PCR)

RNA was harvested and isolated using RNeasy Mini Kit (QIAGEN) according to the manufacturer's instructions. cDNA was synthesised using High Capacity cDNA Reverse Transcription kit (Applied Biosystems), according to the manufacturer's instructions followed by RNase H (Promega) treatment for 30 min at 37°C. *Il10*, *Il12a*, *Ifnb1, Oas1g, Stat1, Stat3, Irf7, Irf9* and *Tlr4* gene expression were quantified by qRT-PCR (7900HT, Applied Biosystems) using the TaqMan system, and normalised to *Hprt1* mRNA. Primer probes used were *Il10* (Mm00439616_m1); *Il12a* (Mm00434165_m1); *Ifnb1* (Mm00439552_s1); *Oas1g* (Mm01730198_m1); *Stat1* (Mm_00439518_m1); *Stat3* (Mm_01219775_m1); *Irf7* (Mm_00516793_g1); *Irf9* (Mm_00492679_m1); *Tlr4* (Mm0045273_m1); *Hprt1* (Mm00446968_m1), all purchased from Applied Biosystems. For the quantification of premature *Il10* mRNA, the following primers were designed using Primer Express 2.0 software and custom made by Applied Biosystems – forward (exon 3) 5'AGCATGGCCCAGAAATCAAG-3'; probe (exon 3) 5'CTCAGGATGCGGCTGA-3'; reverse (intron 4) 5'AGAACGCATCTGCTACTCACACA-3'.

FACS staining

For FACS analysis BMDMs were stimulated with LPS, washed and blocked with anti-CD16/CD32 antibody. Cells were then stained with PE-labeled anti-mouse TLR4 (SA15-21, Bio Legend) for 30 min at 4°C and acquired using BD LSR II (Becton and Dickinson). Data were analysed by FlowJo software.

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Microarray processing and analysis

RNA quality was confirmed (RNA integrity number (RIN) range 9.0-10.0) using an Agilent 2100 Bioanalyzer (Agilent Technologies). RNA was prepared for microarray analysis using the Illumina®TotalPrep-96 RNA Amplification kit following manufacturer's instructions. 1500 ng cRNA was hybridised onto Illumina BeadChip arrays (MouseWG-6 v2) and scanned by an Illumina iScan. Signal intensity calculations and background subtraction were performed using GenomeStudio software (Illumina). Analysis of microarray data was done using GeneSpring GX software version 12.6.1 (Agilent Technologies). A lower threshold of signal intensity was set to 10, the expression values log transformed (base2) and scaled to the 75th percentile for normalisation. The expression value of each gene probe was then normalised to the median of expression of that gene probe in all samples. Gene probes were quality filtered for those 'present' ($p<0.01$) in at least one sample, 19191 gene probes remained. Further statistical analysis and generation of gene lists is described in the relevant figure legends. Canonical pathway analysis was conducted using Ingenuity Pathway Analysis (IPA) software (Ingenuity® Systems, www.ingenuity.com). Expression data conform to the MIAME standards for microarray analysis. Microarray data has been deposited at the Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo/), accession number GSE79809.

Determination of mRNA stability

BMDMs were stimulated with LPS, and 1 h later 10 μg/ml Actinomycin D (*Streptomyces sp.*, Sigma) was added (t = 0) to the cultures. mRNA was harvested after 30, 60 or 90 min reverse transcribed into cDNA and quantified by qRT-PCR.

Quantification of interferon regulator factor 3 (IRF3) activation

Nuclear extracts of 2 h heat-killed *B. pseudomallei* stimulated BMDMs (5:1 *B. pseudomallei*:BMDM) were prepared with the Nuclear Extract kit and assayed with the TransAM IRF3 kit (both from Active Motif) according to the manufacturer's instructions.

Western blotting

BMDMs were rested in 1% FCS for 20 h prior to stimulation with LPS or recombinant IFN-β. At the indicated time points cells were washed with PBS and lysed in RIPA buffer as previously described (35) or in Triton lysis buffer (for IRF3 western blots). Proteins were resolved on a 12.5% SDS-polyacrylamide gel and transferred to PVDF membranes (Millipore). Membranes were probed with antibodies against phospho-ERK1/2 (T185/Y187), total ERK1/2 (both Invitrogen), phospho-p38 (T180/Y182), total p38, phospho-STAT1 (Y701), total STAT1, phospho-IRF3 (S396, 4D4G) (Cell Signalling Technology), total IRF3, GAPDH (FL-335), HSP90α/β (H-114) (Santa Cruz Biotechnology) or Actin (Calbiochem) followed by HRP conjugated goat anti-rabbit IgG, rabbit anti-goat IgG (Southern Biotech) or goat anti-mouse IgM (Calbiochem), and visualised using Pierce ECL western blotting substrate (Thermo Scientific) or Luminata™ Crescendo Western Chemiluminescent HRP Substrate (Millipore). Western blots were quantified using the Quantity One software.

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Statistical analysis

GraphPad Prism software was used to analyse data by one- or two-way ANOVA with Bonferroni's multiple comparison testing, or Student's t-test. Statistical analysis of microarray data was done using GeneSpring GX software version 12.6.1 (Agilent Technologies) and is described in the relevant figure legend.

Results

B. pseudomallei, LPS and Pam3CSK4 induce higher levels of IL-10 but lower levels of proinflammatory cytokines in C57BL/6 compared to BALB/c macrophages

To better understand the factors regulating the production of pro- and anti-inflammatory cytokines in innate immune cells, we investigated TLR-induced IL-10, IL-12, TNF-α and IL-1β production from C57BL/6 and BALB/c macrophages. C57BL/6 and BALB/c macrophages were stimulated over a time-course with either *B. pseudomallei,* which activates both TLR2 and TLR4 signalling (36–38), or purified TLR2 and TLR4 ligands Pam3CSK4 and LPS, respectively (Figure 1). Significantly higher levels of IL-10 were produced by C57BL/6 as compared to BALB/c macrophages with all three stimuli across the time-course of stimulation (Figure 1A). IL-12 is a heterodimeric cytokine composed of IL-12p40 and IL-12p35 subunits, which generate the biologically active IL-12p70 (39). In contrast to IL-10, levels of IL-12p40 production were higher in *B. pseudomallei* and LPS stimulated BALB/c compared to C57BL/6 macrophages (Figure 1B). However, the levels of IL-12p40 production were marginally higher in C57BL/6 macrophages stimulated with Pam3CSK4 (Figure 1B). The expression of *Il12a* mRNA (which encodes the IL-12p35 subunit) was significantly higher in BALB/c compared to C57BL/6 macrophages stimulated with *B. pseudomallei*, LPS or Pam3CSK4 (Figure 1B). Consistent with this, the level of IL-12p70 production was significantly higher in *B. pseudomallei* stimulated BALB/c compared to C57BL/6 macrophages (Figure 1B), although generally below the limit of detection in LPS or Pam3CSK4 stimulated cells. We also observed higher levels of TNF-α (Figure 1C) and IL-1β (Figure 1D) production from BALB/c compared to C57BL/6 macrophages across the time-course of *B. pseudomallei*, LPS and Pam3CSK4 stimulation. Elevated levels of IL-10 but lower proinflammatory cytokine production was also observed in C57BL/6 relative to BALB/c macrophages when stimulated with a range of doses of *B. pseudomallei*, LPS and Pam3CSK4 (Supplementary Figure 1).

IL-10 has the ability to suppress proinflammatory cytokine production from macrophages (3). To investigate if the low levels of IL-12, TNF-α and IL-1β production from C57BL/6 compared to BALB/c macrophages were due to the much higher levels of IL-10 produced by C57BL/6 macrophages, *B. pseudomallei*, LPS and Pam3CSK4 stimulated C57BL/6 *Il10*$^{-/-}$ and BALB/c *Il10*$^{-/-}$ macrophages were assessed for their levels of IL-12p70, TNF-α and IL-1β production (Supplementary Figure 2). IL-12p70 production was found to be greatly increased in *Il10*$^{-/-}$ macrophages relative to WT cells from both strains of mice, however the levels remained higher in BALB/c compared to C57BL/6 macrophages in response to *B. pseudomallei* or LPS stimulation (Supplementary Figure 2A). In Pam3CSK4 stimulated cells, the increase in IL-12p70 production in IL-10 deficient cells compared to WT was modest, although the levels of IL-12p70 production were the same in C57BL/6 *Il10*$^{-/-}$ and

BALB/c *Il10*$^{-/-}$ macrophages (Supplementary Figure 2A). In contrast, TNF-α production was equivalent in *B. pseudomallei*, LPS and Pam3CSK4 stimulated C57BL/6 *Il10*$^{-/-}$ and BALB/c *Il10*$^{-/-}$ macrophages (Supplementary Figure 2B). IL-1β production remained higher in *B. pseudomallei* and LPS stimulated BALB/c *Il10*$^{-/-}$ compared to C57BL/6 *Il10*$^{-/-}$ macrophages, but was more comparable in Pam3CSK4 stimulated cells (Supplementary Figure 2C). Thus, the differential production of TNF-α by all stimuli, and the differences in Pam3CSK4-induced IL-12p70 and IL-1β were largely explained by IL-10 mediated inhibition. However, the differential production of IL-12p70 and IL-1β in *B. pseudomallei* and LPS stimulated C57BL/6 and BALB/c macrophages, although may in part be explained by IL-10, was still observed in the complete absence of IL-10 suggesting an additional mechanism of inhibition in C57BL/6 macrophages. Additionally, the factors contributing to the differential production of IL-10 itself in C57BL/6 and BALB/c macrophages remained unclear.

B. pseudomallei stimulated C57BL/6 macrophages express higher levels of type I IFN pathway related genes compared to BALB/c macrophages

To investigate further the potential mechanisms underlying differential production of IL-10 and proinflammatory cytokines by TLR4 stimulated C57BL/6 and BALB/c macrophages, we first undertook experiments to address whether this was attributable to differential TLR4 expression or early signalling events downstream of this receptor. Steady state *Tlr4* mRNA expression, TLR4 surface expression and TLR4 endocytosis post-LPS stimulation were the same in C57BL/6 and BALB/c macrophages (Figure 2A and B). Early activation of the MAP kinases ERK1/2 or p38 by LPS was also similar in C57BL/6 and BALB/c macrophages (Figure 2C). These data suggest that the differential cytokine production in BALB/c versus C57BL/6 macrophages was not simply due to differential TLR abundance or immediate signalling downstream of TLR4.

We therefore undertook an unbiased microarray analysis of these cells stimulated with *B. pseudomallei* for 3 and 6 h. At 3 h, 790 genes were found to be differentially regulated by *B. pseudomallei* in C57BL/6 and BALB/c macrophages (Figure 2D). The majority of these genes were up-regulated by *B. pseudomallei* stimulation in both strains of mice and of these, almost all were more strongly up-regulated in C57BL/6 compared to BALB/c macrophages. The genes down-regulated by *B. pseudomallei* stimulation in C57BL/6 macrophages were also down-regulated in BALB/c macrophages, although either more strongly or more weakly in comparison. At 6 h, 2246 genes were found to be differentially regulated by *B. pseudomallei* in C57BL/6 and BALB/c macrophages (Figure 2D), suggesting a reinforcement of differential gene expression over time. These genes included those up- and down-regulated by *B. pseudomallei* stimulation and in contrast to the 3 h time-point, were either more strongly or weakly induced in BALB/c macrophages resulting in a complex profile of gene expression (Figure 2D). Of note, *Il10* (BALB/c low – grey dot highlighted red in Figure 2D, 6 hr post stimulation), *Il12a* and *Tnf* (BALB/c high – grey dots highlighted red in Figure 2D, 6 hr post stimulation) were found to be present at similar relative levels between BALB/c and C57BL/6 macrophages, as observed at the protein level (Figure 1). IL-1β was not found to be significantly differentially expressed at the mRNA level,

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suggesting that this cytokine may be differentially regulated post-transcriptionally in C57BL/6 and BALB/c macrophages.

In order to better understand the biological relationships between the differentially regulated genes, transcripts identified at 3 and 6 h, were subjected to canonical pathway analysis (IPA). Amongst the top 10 pathways significantly associated with the gene lists at each time-point, several were found to relate to type I IFN mediated processes. These included 'Interferon Signalling', 'JAK/STAT Signalling', 'Activation of IRF by cytosolic PRRs' and 'Role of JAK1, JAK2, TYK2 in IFN signalling' at 3 h, and 'Activation of IRF by cytosolic PRRs', 'Role of PKR in IFN induction and antiviral response' and 'Interferon Signalling' at 6 h (Figure 2E, Supplementary Table I). The majority of differentially regulated genes within these type I IFN related pathways, including *Oas1g, Stat1*, *Stat3, Irf7* and *Isgf3g* (IRF9), were more highly expressed in C57BL/6 macrophages (Supplementary Table I) and this was validated by qRT-PCR (Figure 2F). In addition, the dominance of the IFN-inducible signalling pathway genes in C57BL/6 versus BALB/c macrophages was validated in an independent experiment using live *B. pseudomallei* (data not shown). These data suggest that enhanced type I IFN signalling in C57BL/6 compared to BALB/c macrophages may be a fundamental difference in the responses of these cells to *B. pseudomallei* and may explain their differential expression of IL-10 versus proinflammatory cytokines.

BALB/c macrophages show reduced IFN-β production, STAT1 and IRF3 activation compared to C57BL/6 macrophages upon TLR4 stimulation

In keeping with higher expression of type I IFN-inducible genes in C57BL/6 macrophages as shown by our microarray analysis (Figure 2), *Ifnb1* mRNA was more highly expressed in *B. pseudomallei* and LPS stimulated C57BL/6 macrophages compared to BALB/c macrophages (Figure 3A). In both strains, *Ifnb1* mRNA expression peaked at 1 h, returning to near baseline levels by 6 h explaining why in the microarray analysis, which was conducted at later time-points, *Ifnb1* itself was not identified as a C57BL/6 high gene (Supplementary Table I). Protein levels of IFN-β production which peaked at 3 h were also higher in *B. pseudomallei* and LPS stimulated C57BL/6 compared to BALB/c macrophages (Figure 3B). We additionally assessed the phosphorylation of STAT1, activated downstream of the type I IFN receptor, in LPS stimulated C57BL/6, BALB/c and C57BL/6 *Ifnar1*$^{-/-}$ macrophages (Figure 3C and D). STAT1 was phosphorylated in macrophages from both strains of mice but was reduced in BALB/c macrophages compared to C57BL/6 after 2 h of stimulation. Thus, the level of type I IFN produced by BALB/c macrophages is sufficient to activate STAT1, but not to the level seen in C57BL/6 macrophages. The total absence of STAT1 phosphorylation in LPS stimulated C57BL/6 *Ifnar1*$^{-/-}$ macrophages confirmed that the STAT1 activation in WT macrophages was due to type I IFN signalling (Figure 3C and D).

To investigate the mechanism by which *B. pseudomallei* induces IFN-β production, C57BL/6 *Trif*$^{-/-}$ and C57BL/6 *Tlr4*$^{-/-}$ macrophages were assessed for their production of IFN-β in response to *B. pseudomallei* or LPS (Figure 3E). In the absence of TRIF or TLR4, neither *B. pseudomallei* nor LPS were able to induce IFN-β production suggesting that differential production of this cytokine in *B. pseudomallei* or LPS stimulated C57BL/6 and

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BALB/c macrophages may be a result of differential activation of the TLR4/TRIF pathway. In support of this, the activation of IRF3 which is required for IFN-β production downstream of TLR4 (40), was lower in *B. pseudomallei* stimulated BALB/c macrophages (Figure 3F-H).

Type I IFN signalling mediates the higher level of IL-10 production observed in *B. pseudomallei* and LPS stimulated C57BL/6 compared to BALB/c macrophages

Type I IFN has been reported to positively regulate IL-10 production in macrophages stimulated with LPS or infected with bacteria such as *Listeria monocytogenes* or *Mycobacterium tuberculosis* (*Mtb*) (11, 16, 33, 41, 42). To determine if autocrine type I IFN signalling mediated the higher level of IL-10 production in C57BL/6 compared to BALB/c macrophages, C57BL/6 *Ifnar1*$^{-/-}$ macrophages were stimulated with *B. pseudomallei* or LPS over a time-course. The level of IL-10 production was significantly reduced in C57BL/6 *Ifnar1*$^{-/-}$ macrophages compared to C57BL/6 WT macrophages, and reduced to the level of IL-10 produced by BALB/c macrophages (Figure 4A). Microarray analysis of C57BL/6 WT, BALB/c and C57BL/6 *Ifnar1*$^{-/-}$ macrophages stimulated with *B. pseudomallei* for 6 h revealed lower expression of *Il10* mRNA and IL-10 pathway genes including *Stat3*, *Jak1, Ccr5, Il4ra* and *Arg2* in BALB/c and C57BL/6 *Ifnar1*$^{-/-}$ macrophages relative to C57BL/6 WT macrophages (Figure 4B). These data support a role for type I IFN in the differential regulation of IL-10 in *B. pseudomallei* and LPS stimulated C57BL/6 and BALB/c macrophages.

Type I IFN signalling promotes IL-10 through active transcription and stabilisation of Il10 mRNA

To investigate the mechanisms by which type I IFN promotes IL-10 production in C57BL/6 macrophages, a detailed time-course of *Il10* mRNA kinetics was carried out on *B. pseudomallei* and LPS stimulated C57BL/6 WT, BALB/c and C57BL/6 *Ifnar1*$^{-/-}$ macrophages. *B. pseudomallei* and LPS stimulated C57BL/6 WT macrophages expressed an initial *Il10* mRNA peak at 0.5 h followed by a second peak at 4 h (Figure 5A). BALB/c macrophages expressed the initial peak of *Il10* mRNA, although at a lower magnitude to C57BL/6 WT macrophages, however completely lacked the second peak of *Il10* mRNA (Figure 5A). In C57BL/6 *Ifnar1*$^{-/-}$ macrophages, while the first peak of *Il10* mRNA was mostly unaffected by the absence of type I IFN signalling, the second peak was completely abrogated (Figure 5A). This suggests that autocrine type I IFN activates a late transcriptional wave of *Il10* mRNA expression, supported by the presence of a second peak of premature *Il10* mRNA, indicative of active transcription, in LPS stimulated C57BL/6 but not BALB/c macrophages (Figure 5B). Additionally, whereas in BALB/c and C57BL/6 *Ifnar1*$^{-/-}$ macrophages a rapid decay of the *Il10* mRNA is observed after 0.5 h, in C57BL/6 macrophages this decay is much less pronounced (Figure 5A). Thus, we further investigated if there was an additional effect of type I IFN on *Il10* mRNA stability. We observed reduced *Il10* mRNA stability in C57BL/6 *Ifnar1*$^{-/-}$ macrophages compared to C57BL/6 WT macrophages demonstrating that autocrine type I IFN also has a stabilising effect on *Il10* mRNA (Figure 5C).

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It has been reported that in LPS stimulated macrophages, type I IFN induced IL-27 is required for the optimal enhancement of IL-10 by type I IFN (43). However, others have shown that murine macrophages stimulated with LPS are unresponsive to IL-27 (44). To investigate if the type I IFN mediated enhancement of IL-10 production in C57BL/6 WT macrophages is dependent on IL-27, C57BL/6, BALB/c and C57BL/6 *Ifnar1*$^{-/-}$ macrophages were stimulated with *B. pseudomallei* or LPS and IL-27 production was determined (Supplementary Figure 3A). In response to both stimuli, the overall magnitude of IL-27 production was similar in C57BL/6 and BALB/c macrophages. In C57BL/6 *Ifnar1*$^{-/-}$ macrophages, IL-27 production was drastically reduced (Supplementary Figure 3A), in agreement with previous studies (45) showing a role for type I IFN in the promotion of IL-27 in C57BL/6 macrophages. However, *B. pseudomallei* and LPS stimulated C57BL/6 *Il27ra*$^{-/-}$ macrophages revealed no significant difference in IL-10 production as compared to WT macrophages (Supplementary Figure 3B). In addition, IL-10 production was similarly enhanced by addition of exogenous IFN-β in WT C57BL/6 and C57BL/6 *Il27ra*$^{-/-}$ macrophages (Supplementary Figure 3C). Furthermore, the addition of exogenous IL-27 did not enhance IL-10 production by LPS stimulated C57BL/6, BALB/c or C57BL/6 *Ifnar1*$^{-/-}$ macrophages (Supplementary Figure 3D). These data demonstrate that type I IFN mediated enhancement of IL-10 in *B. pseudomallei* and LPS stimulated C57BL/6 macrophages is independent of IL-27.

Addition of type I IFN enhances IL-10 production in both C57BL/6 and BALB/c macrophages stimulated with *B. pseudomallei* and LPS

To test whether the addition of type I IFN could enhance IL-10 production in BALB/c macrophages and potentially rescue IL-10 production in this strain, we treated C57BL/6 and BALB/c macrophages with exogenous IFN-β in the presence or absence of PRR ligation by *B. pseudomallei* or LPS (Figure 5D). Treatment with IFN-β in the absence of PRR stimulation did not induce IL-10 production from C57BL/6 or BALB/c macrophages. In *B. pseudomallei* stimulated cells, the addition of IFN-β greatly enhanced IL-10 production in C57BL/6 and BALB/c macrophages, however IL-10 production remained significantly higher in C57BL/6 macrophages (Figure 5D). In LPS stimulated macrophages, the addition of IFN-β also enhanced IL-10 production in both C57BL/6 and BALB/c macrophages (Figure 5D). Furthermore, upon treatment with IFN-β, the levels of LPS induced IL-10 from BALB/c macrophages were as high as that from C57BL/6 macrophages (Figure 5D). BALB/c macrophages therefore have the capacity to produce enhanced levels of IL-10 in response to IFN-β treatment. Furthermore, in LPS stimulated cells, IFN-β had the potential to fully restore IL-10 production to the level observed in similarly stimulated C57BL/6 macrophages.

Type I IFN stimulates IL-10 production in C57BL/6 macrophages through the activation of ERK1/2 MAP kinase

Our data suggest that type I IFN is an important factor in driving the differential production of IL-10 in C57BL/6 and BALB/c macrophages, however, how type I IFN regulates IL-10 production in macrophages is incompletely understood. ERK1/2 and p38 MAP kinases are central regulators of IL-10 in TLR stimulated C57BL/6 macrophages Reviewed in (46). We compared the contribution of these MAP kinases to IL-10 production in C57BL/6 and

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BALB/c macrophages by incubating the cells with p38 and MEK1/2 (upstream of ERK1/2) inhibitors. In keeping with previous studies Reviewed in (46) we observed reduced IL-10 production in LPS stimulated C57BL/6 macrophages upon inhibition of p38 or ERK1/2 signalling (Figure 6A). IL-10 production was further reduced when p38 and ERK1/2 activation were concomitantly blocked (Figure 6A). In BALB/c macrophages, however, IL-10 production was almost completely abrogated by inhibition of p38 signalling alone, but not affected by ERK1/2 inhibition (Figure 6A). Similarly, in LPS stimulated C57BL/6 *Ifnar1*$^{-/-}$ macrophages, inhibition of p38 signalling significantly reduced IL-10 production whereas ERK1/2 inhibition had no effect (Figure 6B). This implied a role for ERK1/2 in the regulation of IL-10 only in the presence of type I IFN. Supporting this, ERK1/2 phosphorylation was induced in C57BL/6 and BALB/c macrophages upon treatment with recombinant IFN-β and this was not observed in C57BL/6 *Ifnar1*$^{-/-}$ macrophages demonstrating the specificity of the IFN-β activation of ERK1/2 (Figure 6C) and that this was not through activation of any PRR. To formally establish the role of p38 and ERK1/2 in the regulation of IL-10 by type I IFN, we inhibited these MAP kinases 1 h before, or 2 h after LPS stimulation, the latter time-point representing the peak of autocrine type I IFN signalling (Figure 3C). Inhibition of p38 prior to LPS stimulation led to a decrease in *Il10* mRNA expression at all time-points, however the type I IFN-dependent second peak of *Il10* mRNA remained present (Figure 6D). Inhibition of p38 at 2 h post-stimulation had no significant effect on *Il10* mRNA levels (Figure 6D). Inhibition of ERK1/2 signalling at either time-point had no effect on the first peak of *Il10* mRNA, while completely blocking the second peak of *Il10* mRNA transcription (Figure 6E). This result was confirmed using an additional structurally unrelated yet specific MEK1/2 inhibitor (Trametinib, Selleckchem, data not shown). These findings demonstrate a requirement for ERK1/2 in the induction of *Il10* mRNA expression by autocrine type I IFN in C57BL/6 macrophages.

Reduced levels of IL-1β and IL-12 production observed in C57BL/6 as compared to BALB/c macrophages result from type I IFN action through IL-10 dependent and independent mechanisms

Since IL-10 mediated inhibition did not fully account for differential IL-1β production in *B. pseudomallei* or LPS stimulated C57BL/6 and BALB/c macrophages (Supplementary Figure 2C), we sought to investigate if type I IFN had an additional effect on the regulation of IL-1β in these cells. Assessment of IL-1β production in *B. pseudomallei* and LPS stimulated C57BL/6 *Ifnar1*$^{-/-}$ macrophages revealed significantly elevated IL-1β production relative to C57BL/6 WT macrophages, to levels similar to BALB/c macrophages (Figure 7A). Indeed, the addition of exogenous IFN-β significantly reduced IL-1β production in both C57BL/6 and BALB/c macrophages, supporting a role for type I IFN in the negative regulation of IL-1β in this system (Figure 7B). To determine if type I IFN could negatively regulate IL-1β production through mechanisms other than through the promotion of IL-10 as previously reported (14, 16, 42), IL-1β production was assessed from C57BL/6, BALB/c and C57BL/6 *Ifnar1*$^{-/-}$ macrophages stimulated with *B. pseudomallei* or LPS in the presence of a blocking antibody against the IL-10 receptor (anti-IL10R), or isotype control antibody (Figure 7C). The blockade of IL-10 signalling enhanced IL-1β production in all cell types. However, in the presence of anti-IL10R, IL-1β production was highly elevated in C57BL/6 *Ifnar1*$^{-/-}$ macrophages relative to C57BL/6 WT and even BALB/c macrophages (Figure 7C). Thus,

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the loss of type I IFN signalling can enhance IL-1β production in the absence of IL-10 signalling, supporting a role for IL-10 independent inhibition of IL-1β production by type I IFN in *B. pseudomallei* and LPS stimulated C57BL/6 macrophages. The production of TNF-α in C57BL/6 *Ifnar1*$^{-/-}$ macrophages was enhanced relative to C57BL/6 WT in the context of *B. pseudomallei* stimulation, but was not affected in LPS stimulated macrophages, implying stimulus-specific regulation of this cytokine by type I IFN (Supplementary Figure 3E).

Type I IFN has been reported to modulate the levels of IL-12 production in various contexts (15, 16, 18, 19, 33). We observed that IL-12p70 production and *Il12a* expression levels were not different in *B. pseudomallei* and LPS stimulated C57BL/6 *Ifnar1*$^{-/-}$ macrophages as compared to C57BL/6 WT macrophages (Figures 8A and B). However, the addition of IFN-β abrogated the production of IL-12p70 from *B. pseudomallei* stimulated cells (no IL-12p70 was detected in LPS stimulated cells), even in BALB/c macrophages, suggesting that type I IFN does have the capacity to inhibit IL-12p70 production in this system (Figure 8C). We considered that residual IL-10 production in C57BL/6 *Ifnar1*$^{-/-}$ macrophages was preventing the induction of high levels of IL-12p70 production, however even in the presence of anti-IL10R, IL-12p70 production was equivalent in C57BL/6 *Ifnar1*$^{-/-}$ macrophages compared to C57BL/6 WT macrophages stimulated with *B. pseudomallei* or LPS (Figure 8D).

Despite a negative regulatory effect of exogenous IFN-β addition on the production of IL-12p70 (Figure 8C), previous studies have indicated that low level basal IFN-β production, which gives rise to tonic type I IFN signalling, is required for optimal IL-12 production from innate cells (16, 18). As tonic type I IFN signalling is absent from C57BL/6 *Ifnar1*$^{-/-}$ macrophages, we postulated that this may contribute to poor IL-12p70 production, even in the absence of IL-10 signalling (Figure 8D). Thus, to uncouple tonic type I IFN signalling from PRR induced autocrine type I IFN signalling, we treated WT C57BL/6 and BALB/c macrophages with anti-IFNAR1 in the presence and absence of anti-IL-10R mAbs (Figure 8E). Upon treatment with anti-IFNAR1, no effect was observed on IL-12p70 production from *B. pseudomallei* or LPS stimulated C57BL/6 macrophages, however, a small increase in IL-12p70 production was observed in BALB/c macrophages (Figure 8E, inset).

As expected, treatment with anti-IL-10R mAbs significantly increased IL-12p70 production in macrophages from both strains of mice, but IL-12p70 remained higher in BALB/c macrophages. In C57BL/6 macrophages, anti-IFNAR1 treatment in the presence of anti-IL-10R, significantly increased IL-12p70 levels relative to anti-IL-10R treatment alone. In contrast, anti-IFNAR1 plus anti-IL-10R mAbs treated BALB/c macrophages did not further enhance IL-12p70 production. Importantly, the level of IL-12p70 production from anti-IFNAR1/anti-IL-10R treated C57BL/6 and BALB/c macrophages were comparable. Similar results were obtained by treating C57BL/6 *Il10*$^{-/-}$ and BALB/c *Il10*$^{-/-}$ macrophages with anti-IFNAR1 (Supplementary Figure 3F). These findings demonstrate that deficient IL-12p70 production from C57BL/6 macrophages can be rescued by the elimination of both PRR-induced IL-10 and type I IFN signalling.

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Discussion

The balance of pro- and anti-inflammatory immune responses is essential to ensure effective but safe pathogen clearance. C57BL/6 and BALB/c mice differ significantly in their immune responses during infections and inflammatory diseases (21, 23, 32). A clear example of this is the *B. pseudomallei* infection model where C57BL/6 mice have enhanced resistance compared to BALB/c mice (26–28). The higher production of proinflammatory cytokines in BALB/c mice has been associated with exacerbated *B. pseudomallei* induced pathology (28–30). However, it is unclear whether this exacerbated pathology is contributed to by decreased control of the pathogen, or an inability to induce a regulated response. In *in vivo* settings, signals from multiple cell types are integrated to induce a balanced production of proinflammatory versus anti-inflammatory cytokines such as IL-10, to control pathogens with minimum host damage. This complexity makes the clear dissection of these mechanisms prohibitive in *in vivo* models. We report here, using an *in vitro* model that allows the examination of these complex phenotypes, that C57BL/6 macrophages produced higher levels of IL-10, but lower levels of TNF-α, IL-12p70 and IL-1β compared to BALB/c macrophages when stimulated with the bacterium *B. pseudomallei*, LPS or Pam3CSK4. We reveal a central role for autocrine type I IFN in the increased production of IL-10 and increased *Il10* mRNA stability by C57BL/6 macrophages, which is accompanied by increased STAT1 and IRF3 activation. The enhanced and prolonged expression of *Il10* was dependent on type I IFN-induced late ERK1/2 phosphorylation. Conversely, type I IFN suppressed the production of the proinflammatory cytokines, IL-12 and IL-1β, via IL-10-dependent and independent mechanisms in *B. pseudomallei* and LPS stimulated C57BL/6 macrophages. These findings demonstrate that fundamental differences in type I IFN induction and function in C57BL/6 and BALB/c macrophage responses may contribute to their differential phenotypes.

The differential production of cytokines by macrophages from both mouse strains was not due to different levels of *Tlr4* mRNA expression or protein production, and this was corroborated by similar levels of early p38 and ERK1/2 activation following LPS stimulation. A comparative microarray analysis of temporal gene expression in *B. pseudomallei* stimulated C57BL/6 and BALB/c macrophages revealed major differences in gene expression, with 790 genes being differentially expressed after 3 hours, and strikingly 2246 genes being differentially expressed after 6 hours post-stimulation. This microarray analysis revealed an unexpected higher expression of type I IFN responsive genes and type I IFN pathway genes in C57BL/6 macrophages, including *Oas1g, Stat1, Stat3, Irf7 and Irf9.*This corresponded with higher IFN-β production in *B. pseudomallei* and LPS stimulated C57BL/6 macrophages. Our findings that *B. pseudomallei* induced IFN-β production was dependent on TLR4 and TRIF, and that IRF3 was more activated in *B. pseudomallei* stimulated C57BL/6 macrophages, suggest that signalling events affecting the TLR4-TRAM/TRIF-TBK1-IRF3 axis, which is critical for the induction of type I IFN downstream of TLR4 (40), may be responsible for the enhanced production of type I IFN in this strain.

Our findings demonstrating higher levels of expression of type I IFN in C57BL/6 macrophages led us to investigate the potential role of type I IFN in differential production of proinflammatory cytokines and IL-10 in C57BL/6 and BALB/c macrophages. Reduced

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IL-10 production in *B. pseudomallei* and LPS stimulated C57BL/6 *Ifnar1*$^{-/-}$ macrophages demonstrated the importance of autocrine type I IFN in maintaining high levels of IL-10 in C57BL/6 macrophages. This is in agreement with previous studies of type I IFN regulated IL-10 production in TLR4 stimulated macrophages (11, 41, 43). However, our detailed investigation into the mechanisms of *Il10* expression in *B. pseudomallei* and LPS stimulated macrophages clearly showed two, previously undescribed, distinct waves of active *Il10* transcription in C57BL/6 but not BALB/c macrophages, the second of which was completely dependent on type I IFN signalling. We additionally identified a role for autocrine type I IFN in the stabilisation of the *Il10* transcript. Complete absence of the type I IFN dependent second peak of *Il10* mRNA in BALB/c macrophages and the similar level of IL-10 production in BALB/c and C57BL/6 *Ifnar1*$^{-/-}$ macrophages stimulated with *B. pseudomallei* and LPS, provide evidence for our novel finding that type I IFN drives differential production of IL-10 in C57BL/6 and BALB/c macrophages. This was further supported by our microarray analysis revealing reduced expression of several IL-10 pathway genes in *B. pseudomallei* stimulated BALB/c and C57BL/6 *Ifnar1*$^{-/-}$ macrophages relative to C57BL/6 WT. Thus, although our data suggest that BALB/c macrophages retain responsiveness to type I IFN, we show that the autocrine type I IFN mediated feed-forward loop, critical for the maintenance of IL-10 production in TLR4 stimulated C57BL/6 macrophages (11, 41, 43), is absent in BALB/c macrophages. Further, the similar levels of IL-10 production in C57BL/6 and BALB/c macrophages stimulated with LPS in the presence of IFN-β suggest that the low level of type I IFN production, as opposed to deficient responsiveness to type I IFN, is a key contributing factor to the reduced levels of IL-10 production observed in BALB/c macrophages.

To date the mechanisms of IL-10 regulation by type I IFN are incompletely understood. In accordance with the literature (35, 47–51) we show here that IL-10 production by C57BL/6 macrophages in response to TLR ligation required both p38 and ERK1/2 activation, although BALB/c macrophages showed less of a requirement for ERK1/2 activation. Our further findings that inhibition of ERK1/2 signalling also has no effect on IL-10 production in macrophages from C57BL/6 *Ifnar1*$^{-/-}$ mice, and that type I IFN can directly induce ERK1/2 phosphorylation, indicate that ERK1/2 is an important factor in the regulation of IL-10 by type I IFN. In accordance with this, we show prolonged induction of *Il10* mRNA expression to be dependent on ERK1/2 but not p38 activation.

It has been reported that type I IFN requires the production of IL-27 to optimally enhance IL-10 production in C57BL/6 mouse macrophages stimulated with LPS (43). We herein found no role for IL-27 signalling in the production of IL-10 by LPS-stimulated macrophages or the enhancement of IL-10 by IFN-β treatment, although IL-27 production itself was dependent on Type I IFN signaling. Our findings thus differ from those of Iyer *et al.* (43) regarding the ability of IL-27 to induce IL-10 production in C57BL/6 macrophages, perhaps due to differences in BMDM culture or laboratory conditions. However, our results are consistent with previous reports that resting and TLR stimulated murine macrophages are unresponsive to IL-27 (16, 44) and that IL-27 does not regulate IL-10 production in *Mtb* infected macrophages (16). Our findings therefore demonstrate that differential production of IL-10 in C57BL/6 and BALB/c macrophages is due a type I IFN dependent but IL-27 independent mechanism.

The assessment of IL-10 deficient C57BL/6 and BALB/c macrophages showed that the differential production of TNF-α, IL-12 and IL-1β in Pam3CSK4 stimulated cells was largely attributable to IL-10. In contrast, only differential TNF-α production was explained by IL-10 in *B. pseudomallei* and LPS stimulated C57BL/6 and BALB/c macrophages. IL-1β has previously been reported to be suppressed by type I IFN in macrophages (14, 16, 42). In the absence of type I IFN signalling, we observed an increase in C57BL/6 macrophage IL-1β production to the level of BALB/c macrophages. In keeping with previous reports (14, 16, 42), negative regulation of IL-1β by type I IFN was retained in the absence of IL-10. Indeed, in the absence of both IL-10 and type I IFN signalling, IL-1β production from C57BL/6 *Ifnar1*$^{-/-}$ macrophages was higher than that of anti-IL10R treated BALB/c macrophages. Thus, C57BL/6 macrophages have a high capacity to produce IL-1β if these two inhibitory loops are removed.

Whereas type I IFN has been shown to negatively regulate macrophage production of TNF-α and IL-1β via mechanisms that are predominantly or only partially dependent on IL-10, respectively (14, 16), it has been reported that type I IFN can both positively and negatively regulate IL-12 production *in vitro* (15–19). Furthermore, the role for IL-10 in the regulation of IL-12 by type I IFN is not fully understood and may depend on the context (15–17). Despite our finding that treatment with exogenous IFN-β can suppress *B. pseudomallei* induced IL-12 production, IL-12p70 production was not enhanced in C57BL/6 *Ifnar1*$^{-/-}$ macrophages compared to C57BL/6 WT macrophages, even in the presence of anti-IL-10R mAb. We postulated that the failure of C57BL/6 *Ifnar1*$^{-/-}$ macrophages to produce high levels of IL-12 was due to the absence of tonic type I IFN signalling, reported to be required for optimal IL-12 production in innate cells (18). Indeed, blockade of type I IFN signalling with the anti-IFNAR1 antibody only 2 h prior to stimulation showed an increase in IL-12p70 protein in C57BL/6 macrophages when IL-10 signalling was concomitantly blocked. The levels of IL-12p70 in C57BL/6 macrophages were similar to those observed in BALB/c macrophages when type I IFN and IL-10 signalling were simultaneously blocked. Collectively our data show that differential production and function of TLR-induced type I IFN accounts for the differential production of IL-12p70 and IL-1β in C57BL/6 and BALB/c macrophages.

We herein demonstrate distinct profiles of pro- and anti-inflammatory cytokine production in C57BL/6 and BALB/c macrophages and provide mechanisms to account for these differences. C57BL/6 macrophages, in response to TLR4-ligation, produced higher levels of type I IFN accompanied by increased induction of type I IFN-inducible genes and STAT1 activation, as compared to BALB/c macrophages. Type I IFN was found to induce increased and sustained *Il10* mRNA expression via an ERK1/2-dependent pathway, demonstrating the importance of temporal regulation of cytokine gene expression. Additionally, type I IFN increased *Il10* mRNA stability. Collectively, these effects of type I IFN resulted in the increased IL-10 protein production observed in C57BL/6 macrophages. Type I IFN regulated IL-12p70 and IL-1β production via IL-10-dependent and IL-10-independent mechanisms, with both accounting for the differential production of these proinflammatory cytokines by C57BL/6 and BALB/c macrophages. This work highlights the complex role of type I IFN in the regulation of innate immune responses, and further suggests that the extent of type I IFN mediated activity may differ according to the genetic background of the host.

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Our findings emphasise the fact that the C57BL/6 genetic strain of mouse, commonly used in immunological studies, may not reflect the true breadth of immunoregulatory mechanisms operating in TLR stimulated macrophages, or in broader immunological contexts, and supports the dissection of complex molecular mechanisms of cytokine gene regulation in model systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

(*B. pseudomallei* or Bps)
Burkholderia pseudomallei

RU
relative units

WT
wild type

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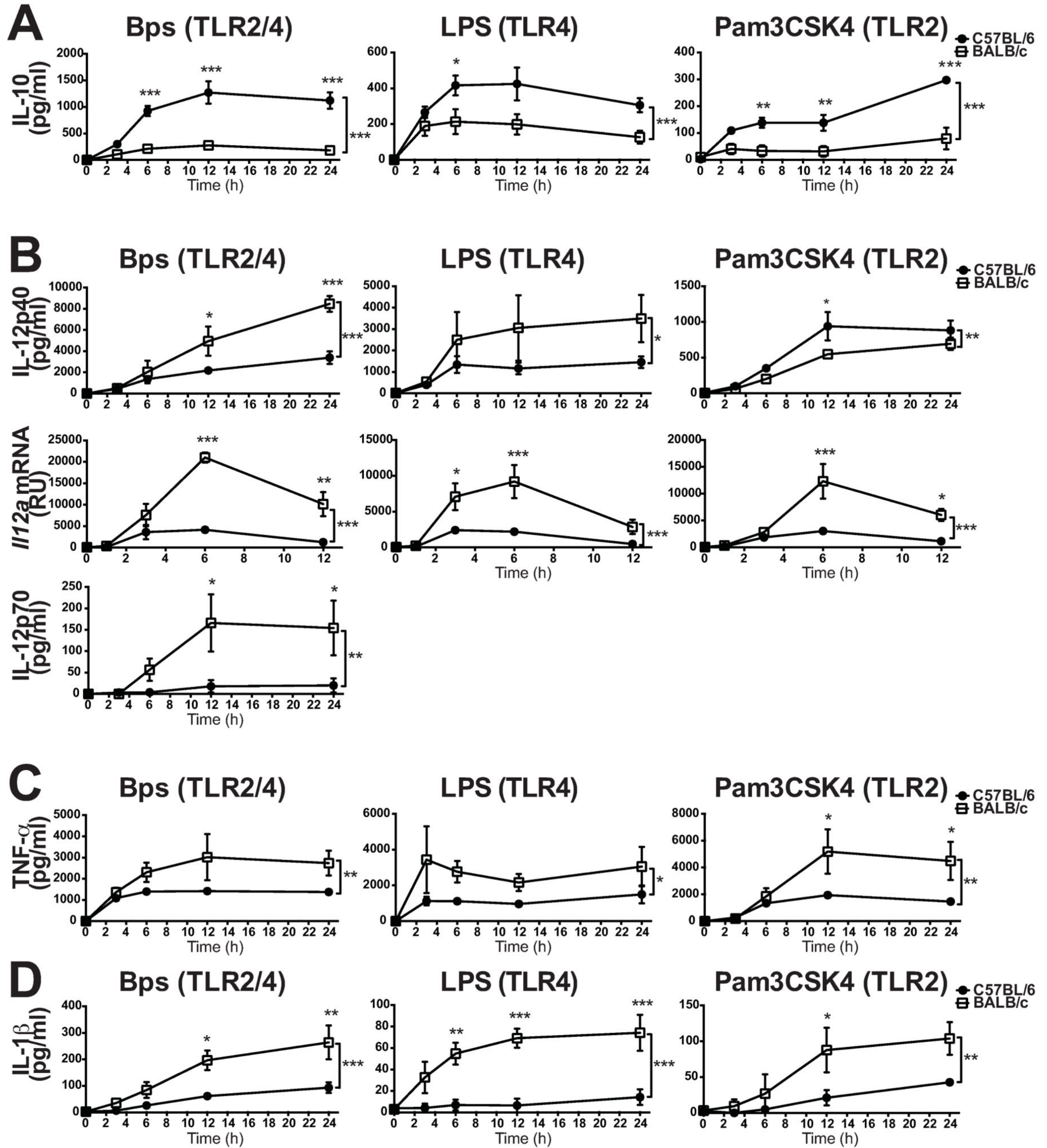

Figure 1. C57BL/6 macrophages produce higher levels of IL-10 but lower levels of proinflammatory cytokines compared to BALB/c macrophages in response to bacterial products.
C57BL/6 and BALB/c BMDMs were stimulated with *B. pseudomallei* (Bps in figure), LPS or Pam3CSK4 for the indicated times. IL-10 (A), IL-12p40, IL-12p70 (B), TNF-α (C) and IL-1β (D) protein levels in supernatants were determined by ELISA. *Il12a* mRNA expression (B) was determined by qRT-PCR and normalised to *Hprt1* mRNA expression. Graphs show means ±SEM of 3 independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$ as determined by two-way ANOVA (Bonferroni's multiple comparison test).

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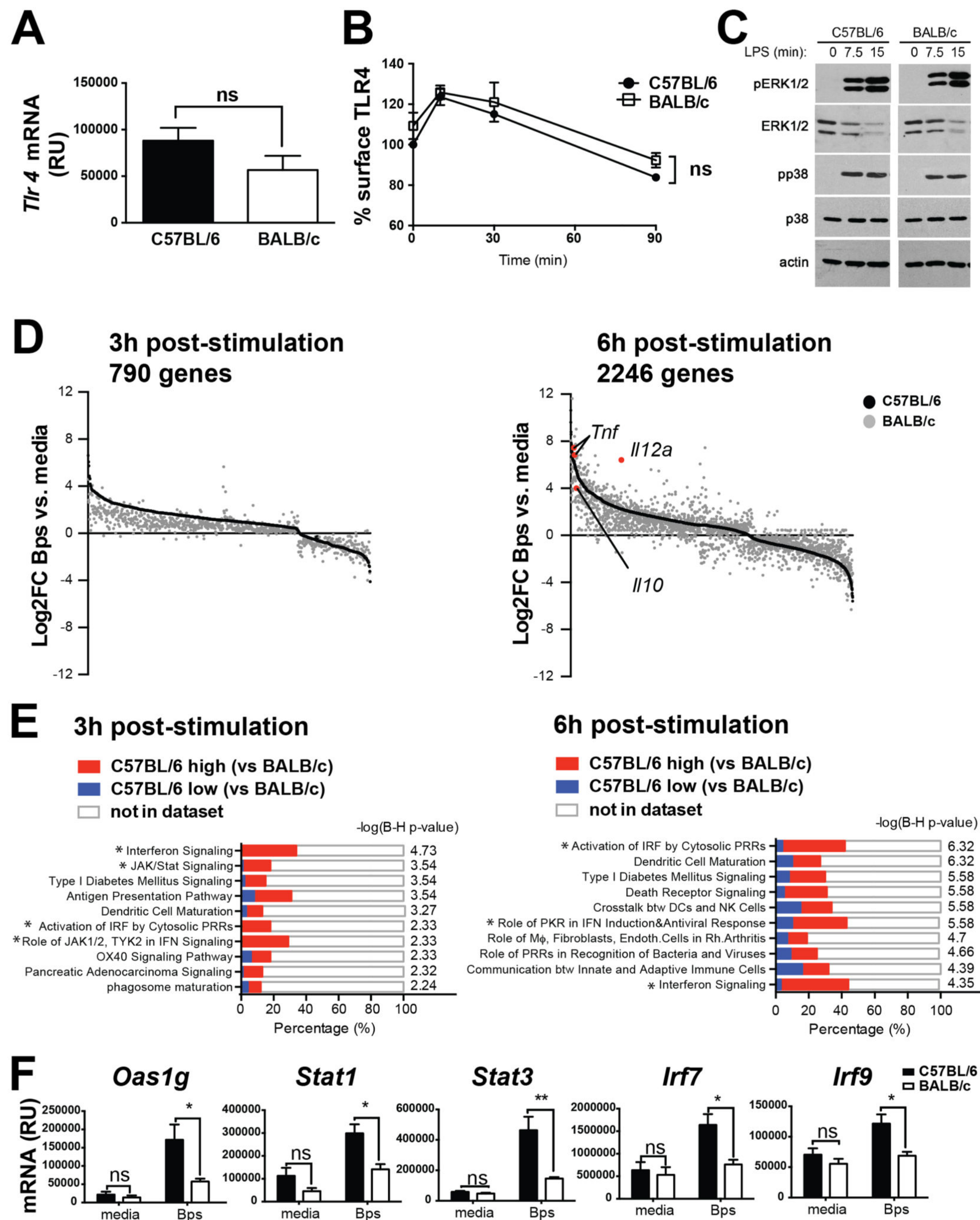

Figure 2. C57BL/6 macrophages show similar early TLR4-induced responses, but higher expression of type I IFN pathway genes, as compared to BALB/c macrophages.

(A) *Tlr4* mRNA expression in C57BL/6 and BALB/c BMDMs at steady state was determined by qRT-PCR and normalised to *Hprt* mRNA expression. (B) C57BL/6 and BALB/c BMDMs were stimulated with LPS for the indicated times and TLR4 expression analysed by flow cytometry. (C) BMDMs were stimulated with LPS as indicated and phosphorylation of ERK1/2 and p38 in whole cell lysates was determined by Western blotting. (D-F) C57BL/6 and BALB/c BMDMs were stimulated with *B. pseudomallei* for 3 h or 6 h in triplicate cultures. Total RNA was isolated and processed for microarray analysis

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as described in Materials and Methods. (D) Genes significantly differentially regulated by *B. pseudomallei* in C57BL/6 and BALB/c BMDMs were identified by two-way ANOVA analysis ($p<0.01$, Benjamini-Hochberg False Discovery Rate) and the selection of genes that were significantly different due to both stimulation and strain. Genes significantly changed due to stimulus alone or strain alone, were excluded from the analysis. Expression level of individual genes (black dots = C57BL/6; grey dots = BALB/c) are shown as Log2 fold change over corresponding media control samples, and ordered according to their C57BL/6 expression level. (E) Top 10 IPA pathways significantly associated with the genes differentially regulated by *B. pseudomallei* stimulation in C57BL/6 and BALB/c macrophages at each time-point are shown. The x-axis represents the percentage overlap between input genes and annotated genes within the pathway. Colours within bars denote genes that are more highly expressed in C57BL/6 or BALB/c macrophages. Benjamini-Hochberg (B-H) corrected –log(*p*-value) represents pathway association. * denotes type I IFN related pathway. (F) C57BL/6 and BALB/c BMDMs were stimulated with *B. pseudomallei* for 6 h. Gene expression was determined by qRT-PCR and expression values normalised to *Hprt1* mRNA expression. All graphs show means ±SEM of 3 independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$ as determined by two-way ANOVA (Bonferroni's multiple comparison test). Western blot (C) is representative of 2 independent experiments.

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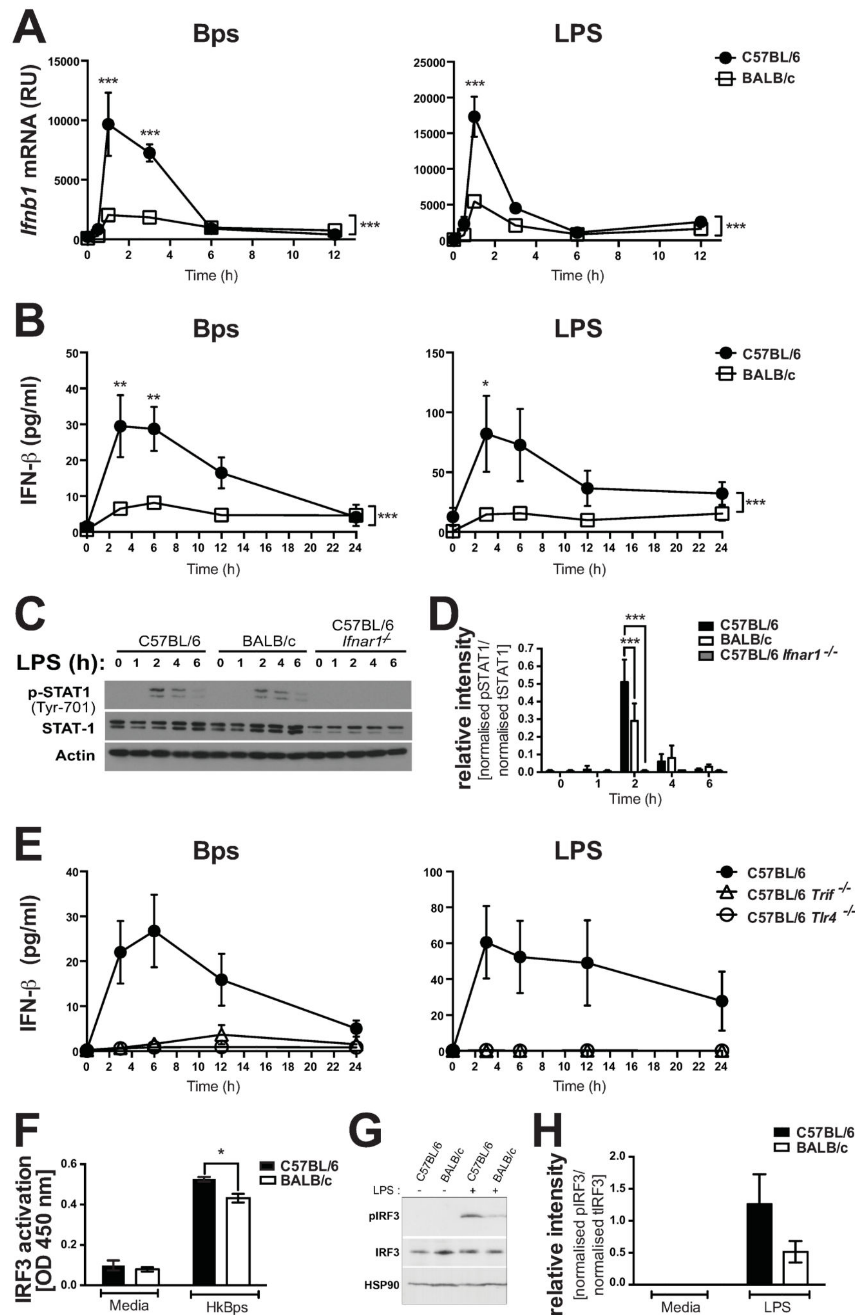

Figure 3. TLR4 dependent IFN-β production, STAT1 and IRF3 activation are higher in C57BL/6 compared to BALB/c macrophages.

BMDMs were stimulated with *B. pseudomallei* or LPS for the indicated times. (A) *Ifnb1* mRNA expression was determined by qRT-PCR and normalised to *Hprt1* mRNA expression. (B) IFN-β production was quantified by ELISA. (C) Whole protein extracts were generated and analysed by Western blot for total and phosphorylated STAT1, and Actin loading control. (D) Relative intensity of 2 independent experiments shown for data represented in C. (E) IFN-β production was quantified by ELISA. (F) C57BL/6 and BALB/c macrophages were stimulated with *B. pseudomallei* for 2 h and nuclear extracts analysed for active IRF3

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by ELISA. (G) Whole protein extracts were generated and analysed by Western blot for total and phosphorylated IRF3, and HSP90 loading control. (H) Relative intensity of 3 independent experiments shown from data in G. Graphs show means ±SEM of 2-4 (E) or at least 3 independent experiments (A, B). *p<0.05, **p<0.01, ***p<0.001 as determined by two-way ANOVA (Bonferroni's multiple comparison test).

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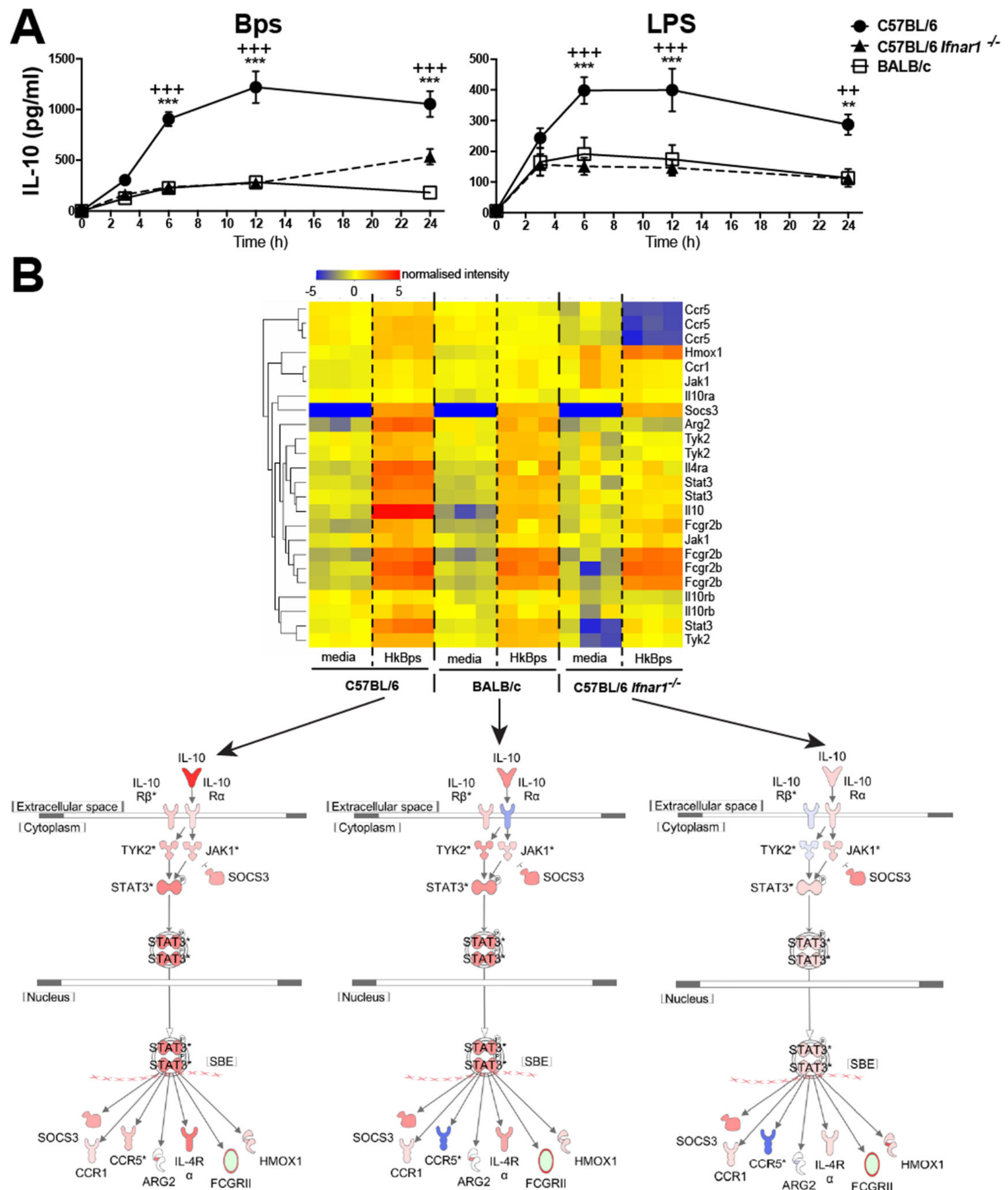

Figure 4. *B. pseudomallei* and LPS stimulated C57BL/6 *Ifnar1*$^{-/-}$ and BALB/c macrophages have similar levels of IL-10 production and show reduced expression of IL-10 pathway genes compared to C57BL/6 WT macrophages.

(A) C57BL/6, BALB/c and C57BL/6 *Ifnar1*$^{-/-}$ BMDMs were stimulated with *B. pseudomallei* or LPS for the indicated times. IL-10 production was quantified by ELISA. Graphs show means ± SEM of 4 independent experiments. **$p<0.01$, ***$p<0.001$ as determined by two-way ANOVA (*C57BL/6 vs. BALB/c; $^{+}$C57BL/6 vs. C57BL/6 *Ifnar1*$^{-/-}$), Bonferroni's multiple comparison test. (B) C57BL/6, BALB/c and C57BL/6 *Ifnar1*$^{-/-}$ BMDMs were stimulated with *B. pseudomallei* for 6 h in triplicate cultures. Total RNA was

isolated and processed for microarray analysis as described in Materials and Methods. Normalised expression of genes annotated within the IL-10 pathway (IPA) are shown hierarchically clustered according to expression. Lower panel shows overlay of gene expression level (red high, blue low) on a schematic of the IL-10 pathway (IPA).

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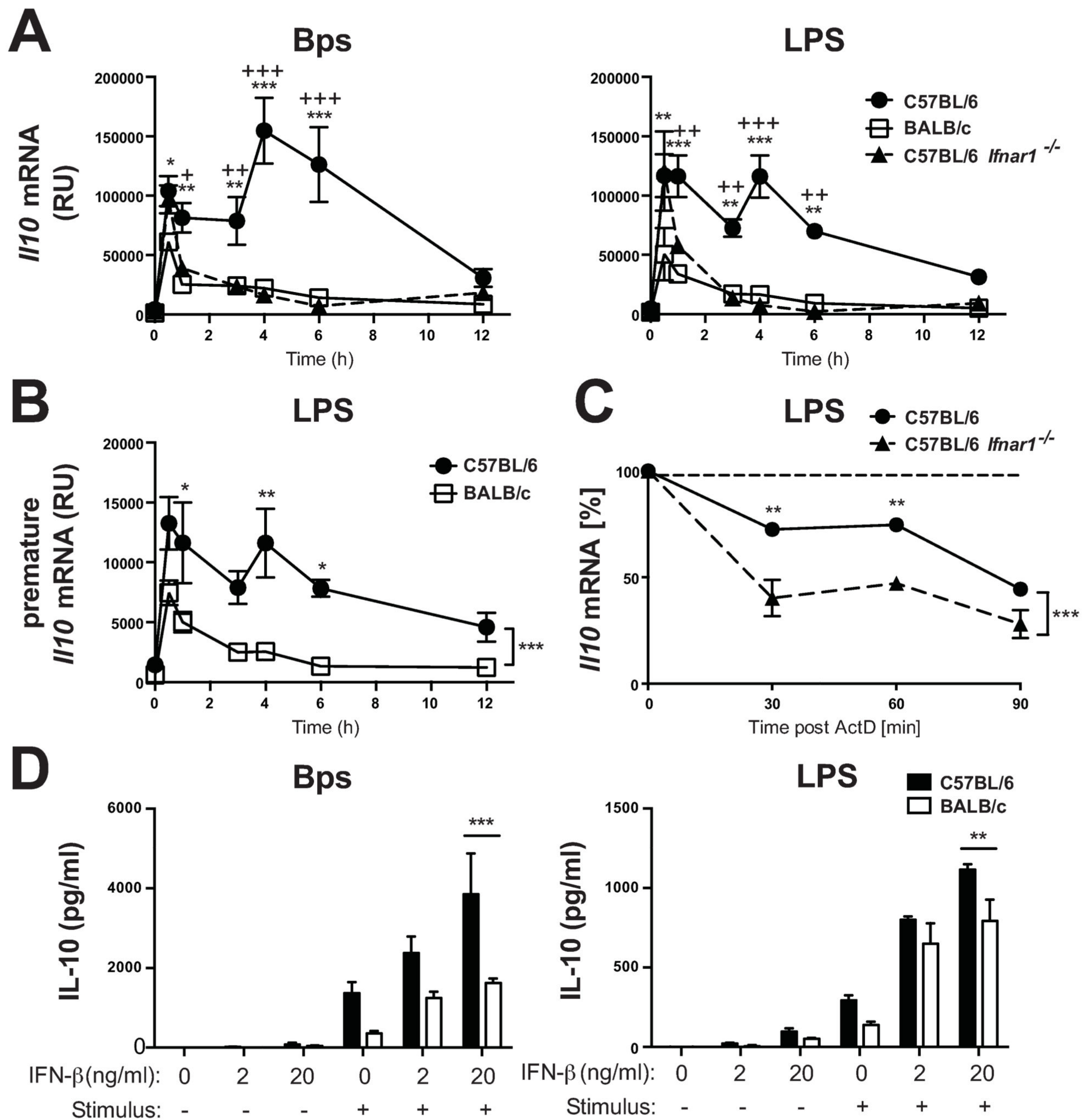

Figure 5. Type I IFN promotes *B. pseudomallei* and LPS stimulated IL-10 production transcriptionally and by stabilisation of *Il10* mRNA in C57BL/6 and BALB/c macrophages.

(A, B) C57BL/6, BALB/c and C57BL/6 *Ifnar1*$^{-/-}$ BMDMs were stimulated with *B. pseudomallei* or LPS for the indicated times. (C) C57BL/6 and C57BL/6 *Ifnar1*$^{-/-}$ BMDMs were stimulated with LPS for 1 h and treated with Actinomycin D (ActD). (A-C) *Il10* mRNA was harvested and quantified by qRT-PCR, normalised to *Hprt1* mRNA levels. (D) C57BL/6 and BALB/c BMDMs were treated with 2 or 20 ng/ml IFN-β for 2 h prior to stimulation with *B. pseudomallei* or LPS for 24 h. IL-10 production was quantified by ELISA. Graphs show means ±SEM of 2-3 independent experiments. *$p<0.05$, **$p<0.01$,

***$p<0.001$, two-way ANOVA (*C57BL/6 vs. BALB/c; $^{+}$C57BL/6 vs. C57BL/6 *Ifnar1*$^{-/-}$), Bonferroni's multiple comparison test.

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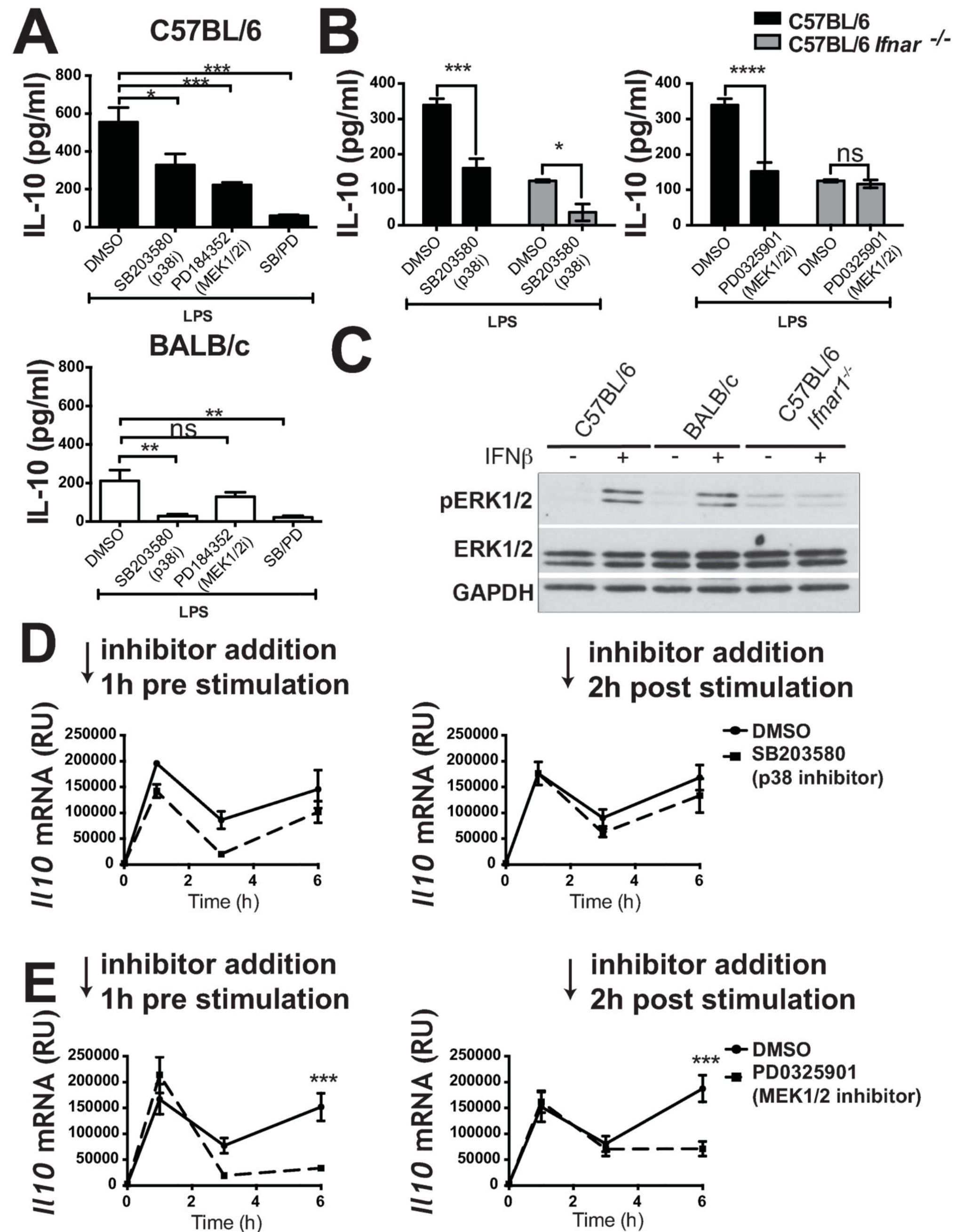

Figure 6. Type I IFN stimulates IL-10 production in C57BL/6 macrophages through the activation of ERK1/2 MAP kinase.

(A) C57BL/6 and BALB/c BMDMs were treated with PD184352 and/or SB203580 or DMSO as control 1 h prior to stimulation with LPS for 24 h. IL-10 production was quantified by ELISA. (B) C57BL/6 WT and *Ifnar*$^{-/-}$ BMDMs were treated with PD0325901 or SB203580 or DMSO as control 1 h prior to stimulation with LPS for 24 h. IL-10 production was quantified by ELISA. (C) C57BL/6 WT, *Ifnar1*$^{-/-}$ and BALB/c BMDMs were stimulated with 20 ng/ml IFN-β. Whole protein extracts were generated and analysed by Western blot for total and phosphorylated ERK1/2 and GAPDH as loading control. (D, E)

C57BL/6 and BALB/c WT BMDMs were treated with SB203580 or PD0325901 1 h prior to or 2 h post stimulation with LPS. *Il10* mRNA was harvested at indicated times and quantified by qRT-PCR, normalised to *Hprt1* mRNA levels. Graphs show means ±SEM of 3-5 independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ns= non-significant as determined by one-way (A) or two-way ANOVA analysis (Bonferroni's multiple comparison test) (B, D, E). Western blot shown is representative of 3 independent experiments.

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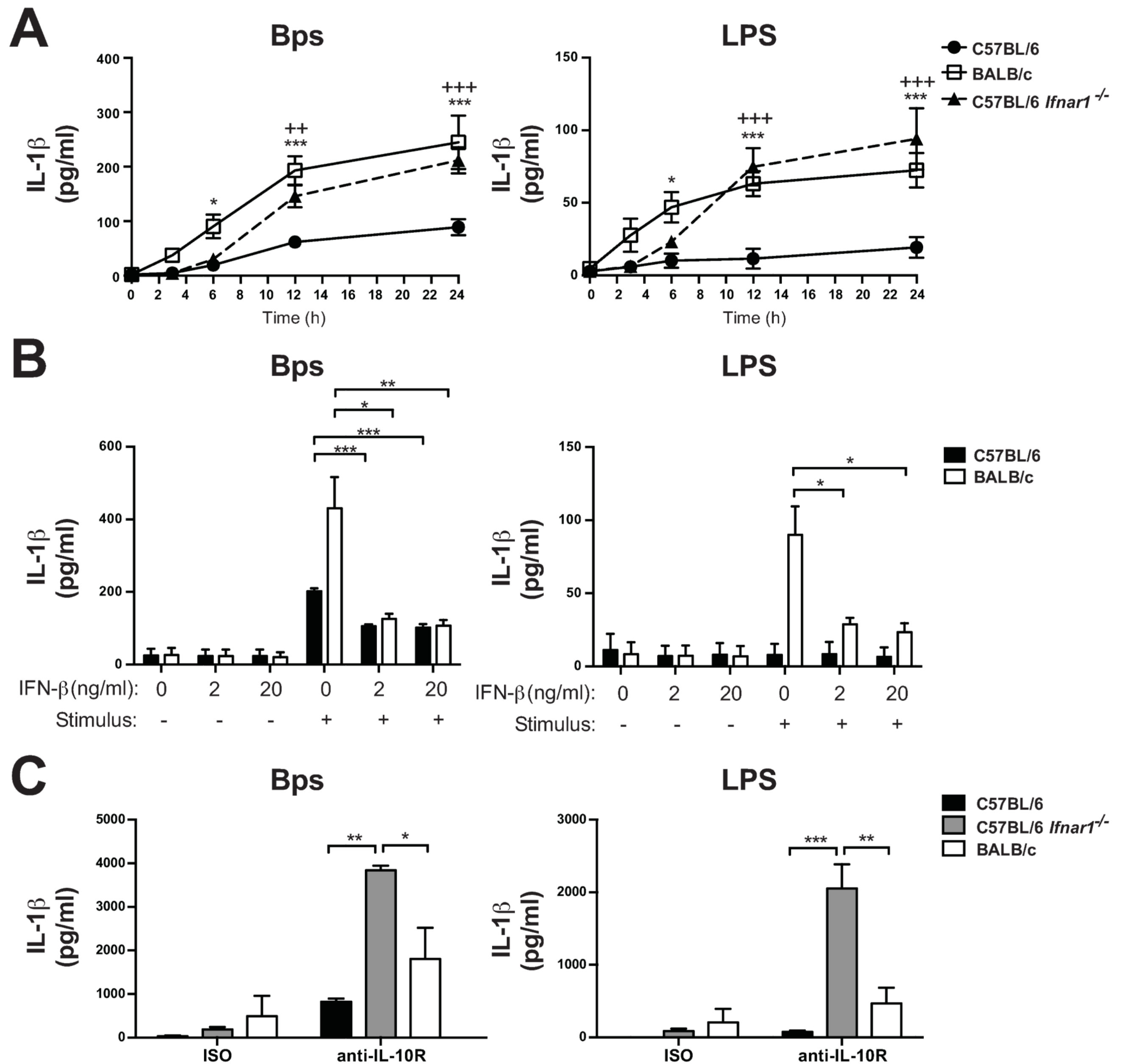

Figure 7. IL-1β production is inhibited in *B. pseudomallei* and LPS stimulated C57BL/6 macrophages by type I IFN through IL-10 dependent and independent mechanisms.

(A) C57BL/6, BALB/c and C57BL/6 *Ifnar1*$^{-/-}$ BMDMs were stimulated with *B. pseudomallei* or LPS for the indicated times. (B) C57BL/6 and BALB/c BMDMs were treated with 2 or 20 ng/ml IFN-β for 2 h prior to stimulation with *B. pseudomallei* or LPS for 24 h. (C) C57BL/6, BALB/c and C57BL/6 *Ifnar1*$^{-/-}$ BMDMs were stimulated with *B. pseudomallei* or LPS for 24 h in the presence of anti-IL10R or isotype control added at the time of stimulation. IL-1β production was quantified by ELISA. Graphs show means ± SEM of 2 (C) or at least 3 (A, B) independent experiments. *p<0.05, **p<0.01, ***p<0.001 as

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determined by one-way (B) or two-way (A,C) ANOVA (*C57BL/6 vs. BALB/c; +C57BL/6 vs. C57BL/6 *Ifnar1*$^{-/-}$), Bonferroni's multiple comparison test.

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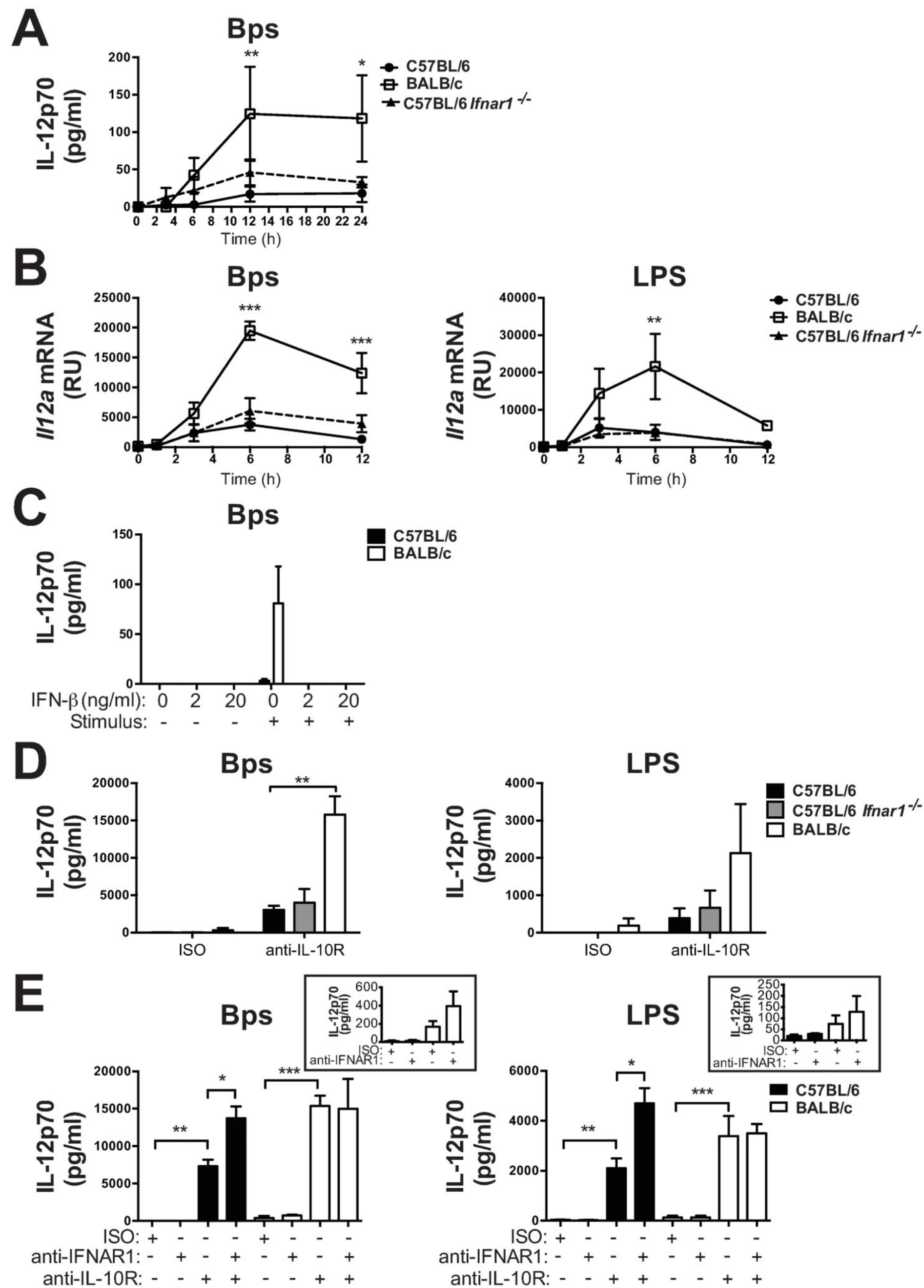

Figure 8. IL-12p70 is negatively regulated by type I IFN and IL-10 in *B. pseudomallei* and LPS stimulated C57BL/6 and BALB/c macrophages.

(A, B) C57BL/6, BALB/c and C57BL/6 *Ifnar1*$^{-/-}$ BMDMs were stimulated with *B. pseudomallei* or LPS for the indicated times. (C) C57BL/6 and BALB/c BMDMs were treated with 2 or 20 ng/ml IFN-β for 2 h prior to stimulation with *B. pseudomallei* for 24 h. (D) C57BL/6, BALB/c and C57BL/6 *Ifnar1*$^{-/-}$ BMDMs were stimulated with *B. pseudomallei* or LPS for 24 h in the presence of anti-IL10R or isotype control added at the time of stimulation. (E) C57BL/6 and BALB/c BMDMs were stimulated with *B. pseudomallei* or LPS for 24 h in the presence of anti-IFNAR1, anti-IL10R or isotype control

added 2 h prior to stimulation. IL-12p70 production was quantified by ELISA and *Il12a* mRNA expression by qRT-PCR. Graphs show means ±SEM of 2 (B,D) or at least 3 (A, C, E) independent experiments. *$p<0.05$, **$p<0.01$, ***$p<0.001$ as determined by two-way ANOVA (Bonferroni's multiple comparison test).

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