

Cell surface IL-1 α trafficking is specifically inhibited by interferon- γ , and associates with the membrane via IL-1R2 and GPI anchors.

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ABSTRACT

Interleukin-1 (IL-1) is a powerful cytokine that drives inflammation and modulates adaptive immunity. Both IL-1 α and IL-1 β are translated as proforms that require cleavage for full cytokine activity and release, while IL-1 α is reported to occur as an alternative plasma membrane-associated form on many cell types. However, the existence of cell surface IL-1 α (csIL-1 α) is contested, how IL-1 α tethers to the membrane is unknown, and signalling pathways controlling trafficking are not specified. Using a robust and fully validated system we show that macrophages present *bona fide* csIL-1 α after ligation of toll-like receptors. Pro-IL-1 α tethers to the plasma membrane in part through IL-1R2 or via association with a Glycosylphosphatidylinositol-anchored protein, and can be cleaved, activated and released by proteases. csIL-1 α requires *de novo* protein synthesis and its trafficking to the plasma membrane is exquisitely sensitive to inhibition by interferon γ , independent of expression level. We also reveal how prior csIL-1 α detection could occur through inadvertent cell permeabilisation, and that senescent cells do not drive the senescent-associated secretory phenotype via csIL-1 α , but rather via soluble IL-1 α . We believe this data is important for determining the local or systemic context in which IL-1 α can contribute to disease and/or physiological processes.

INTRODUCTION

Inflammation defends against acute insults such as infection or injury, but also drives numerous pathologies such as atherosclerosis, obesity, type 2 diabetes, and cancer. Interleukin-1 (IL-1) is an ancient cytokine that regulates both the innate and adaptive immune system[1], with the extended human IL-1 family containing 11 ligands and 10 receptors that both promote and inhibit inflammation. IL-1 α and IL-1 β both signal via the type 1 IL-1 receptor (IL-1R1) and induce identical effects[1], including cytokine secretion, increased adhesion and/or MHC/Costimulatory molecules, vascular leakage[2], T_H17 cell differentiation, and T-cell expansion and survival[3]. In addition, senescent cells utilise IL-1 α to drive the senescence-associated secretory phenotype (SASP)[4-6] that is vital for removal of pre-malignant cells, but mediates deleterious effects when senescent cells accumulate during aging.

Due to these potent activities IL-1 regulation is intensely researched. IL-1 signalling is countered by a receptor antagonist (IL-1RA), a decoy receptor (IL-1R2), and expression of IL-1 α and IL-1 β as pro-proteins that require processing for full activity[7-11]. IL-1 α is cleaved by calpain[10], thrombin[11] and caspase-5[6], which significantly increases activity. IL-1 β is activated by caspase-1 after inflammasome engagement[12]. However, although IL-1 β expression requires stimulation (e.g. via toll-like receptors (TLRs)), IL-1 α is constitutively expressed.

Shortly after the cloning of IL-1[13] a plasma membrane-associated form was reported on mouse macrophages[14] and human monocytes[15]. This cell surface IL-1 (csIL-1) was induced by heat-killed bacteria or lipopolysaccharide (LPS), suggesting an important role in immune responses, and was later shown to be exclusively IL-1 α [16]. However, because IL-1 α does not contain a signal peptide for translocation into the endoplasmic reticulum, or any hydrophobic regions for membrane insertion, the existence of csIL-1 α was refuted [17, 18] and remains controversial. Most studies localising IL-1 α to the cell surface utilised IL-1-dependent bioassays (e.g. thymocyte proliferation) that responded to formaldehyde-fixed cells bearing putative csIL-1 α [14-16, 19, 20]. However, others demonstrated that fixation causes leakage of intracellular IL-1 α , and thus bioassays may have reported this rather than csIL-1 α [17, 18]. In addition, although subsequent studies utilised flow cytometry to directly identify csIL-1 α [4, 16, 21], without a viability dye for a dead cell gate, permeabilised cells could allow antibody binding to intracellular IL-1 α and/or non-specific antigens.

Irrespective of controversies, little is known about the nature of csIL-1 α association with the plasma membrane. Reports show csIL-1 α to be the pro-form[20, 22], with 10% of csIL-1 α phosphorylated compared to 0% of released IL-1 α [22]. D-mannose treatment reduced cell-associated IL-1 activity, and enabled pro-IL-1 α immunoprecipitation from the conditioned media, suggesting csIL-1 α associates via a lectin-like interaction to a glycoprotein[20]. Pro-IL-1 α can be myristoylated at Lys 82/83[23], but although myristoylation can mediate membrane association, if this actually tethers csIL-1 α was not tested. Finally, csIL-1 α does not require inflammasome components (ASC, NLRP3, NLRC4, P2X7) or caspase-1, and is not sensitive to caspase inhibitors[21].

We show that macrophages genuinely present csIL-1 α after TLR ligation, and this requires *de novo* synthesis. csIL-1 α tethering occurs through IL-1R2 and a Glycosylphosphatidylinositol (GPI)-anchored protein, and csIL-1 α can be activated and released after protease cleavage. Importantly, trafficking of IL-1 α to the plasma membrane is exquisitely sensitive to inhibition by interferon γ . Our data suggests that prior csIL-1 α detection may have been due to inadvertent cell permeabilisation and leakage of intracellular IL-1 α . Indeed, we show that senescent cells do not drive the SASP via csIL-1 α [4], but rather via soluble IL-1 α . We believe this novel data is important to understand the local or systemic situations in which IL-1 α can participate to disease and/or physiological processes.

RESULTS

Fixation or absence of a viability dye leads to misidentification of cell surface IL-1 α . As most studies identifying csIL-1 α on macrophages used bioassays requiring fixation[14-16, 19, 20], we investigated if fixation permeabilised cells. Flow cytometry with an anti-IL-1 α monoclonal antibody revealed low levels of IL-1 α staining with unfixed bone marrow-derived macrophages (BMDMs), which was increased to suspiciously high levels after fixation (**Fig 1A**). Furthermore, significant levels of IL-1 α leaked from LPS-treated fixed BMDMs into the conditioned media (**Fig 1B**). A viability dye also showed high levels of macrophage permeabilisation after fixation (**Fig 1C**), which occurred with low concentrations of formaldehyde and increased at higher levels (**Fig 1D**). Finally, using flow cytometry to examine unstimulated BMDMs with anti-IL-1 α and viability dye co-staining revealed minimal IL-1 α stained cells in the 'live' gate, but higher levels of putative IL-1 α in the permeabilised 'dead' cell gate (**Fig 1E**). Importantly, these permeabilised cells inadvertently increased during csIL-1 α staining, as freshly harvested unstained BMDMs were ~90% viable (**Fig 1F**). Together this shows that fixation leads to permeabilisation and IL-1 α leakage from cells, suggesting studies using fixation may misidentify csIL-1 α , and that flow cytometry without a viability gate can report intracellular IL-1 α staining.

LPS induces *bona fide* cell surface IL-1 α on macrophages. Using flow cytometry with a viability gate we tested wild-type (WT) and *Il1a*^{-/-} BMDMs for csIL-1 α with the commonly used anti-IL-1 α ALF-161 clone. Untreated BMDMs showed equivalent basal staining in both groups, while LPS treatment increased csIL-1 α in WT, but not *Il1a*^{-/-} cells (**Fig 2A**). However, permeabilisation revealed high levels of LPS-inducible staining in *Il1a*^{-/-} BMDMs (**Fig 2B**), suggesting non-specificity of ALF-161 anti-IL-1 α . Thus, we repeated experiments with clone BL1a-89 anti-IL-1 α , which revealed low basal staining in LPS-treated *Il1a*^{-/-} BMDMs with or without permeabilisation (**Fig 2C**), proving its specificity for IL-1 α . Together, using flow cytometry, a viability dye and BL1a-89 anti-IL-1 α revealed LPS-treated WT BMDMs to present what we reason to be bona fide csIL-1 α , while *Il1a*^{-/-} BMDMs did not (**Fig 2D & S1**). Longer LPS treatment resulted in more BMDMs presenting csIL-1 α (**Fig 2E**), with csIL-1 α maintained for up to 40h. Testing multiple individual BMDM differentiations with these conditions revealed a range of csIL-1 α levels, with typically 15% of macrophages bearing detectable csIL-1 α (**Fig 2F,G**). csIL-1 α could also be induced by the TLR2 ligands Pam3CSK4 and HKLM, and to a lesser extent by the TLR2/6 ligand FSL-1 and the TLR7 ligand ssRNA40 (**Fig 2H**), with no synergy seen. Finally, presentation of macrophage csIL-1 α required *de novo* protein synthesis, as evidenced by emetine inhibition of TNF α release as a positive control (**Fig 2I**), and emetine inhibition of total IL-1 α and csIL-1 α (**2J,K**). However, csIL-1 α did not require caspase-1 (**Fig S2**). Thus, macrophages express *bona fide* csIL-1 α after TLR ligation. Subsequent csIL-1 α detection used BL1a-89, a viability gate and 16 h of stimulation.

A proportion of cell surface IL-1 α is dependent on the presence of IL-1 receptors. Previous work indicates that csIL-1 α is tethered to the plasma membrane by a lectin-like interaction[20], via phosphorylation[22], or by myristoylation[23]. We investigated whether csIL-1 α could be bound to cell surface IL-1 receptors. Thus, BMDMs were stimulated with LPS to induce csIL-1 α , and then treated with high concentrations of IL-1RA to compete any ligand off the IL-1 receptors. IL-1RA treatment reduced csIL-1 α levels (**Fig 3A**), while IL-1 α treatment did not. Macrophages are reported to express IL-1R2[24], but not IL-1R1[25], while pro-IL-1 α can bind IL-1R2 in the macrophage cytosol[10]. In keeping with this, *Il1r2*^{-/-} BMDMs presented less csIL-1 α after LPS (**Fig 3B**), despite WT and *Il1r2*^{-/-} BMDMs expressing equivalent total IL-1 α (**Fig 3C**), and excess IL-1RA did not reduce csIL-1 α on *Il1r2*^{-/-} BMDMs (**Fig 3D**). Together, this suggests that a proportion of IL-1 α is associated with the plasma membrane via interaction with its cognate receptor IL-1R2.

A proportion of cell surface IL-1 α associates with the membrane via a GPI-anchor. As csIL-1 α didn't drop to baseline after excess IL-1RA or on *Il1r2*^{-/-} BMDMs (**Fig 3**), we investigated how else IL-1 α could associate with the plasma membrane. Previous work shows pro-IL-1 α to be the form associated with the cell surface[20, 22]. Thrombin specifically cleaves pro-IL-1 α N-terminal to the calpain cleavage site[11]. Incubation of LPS-treated BMDMs with thrombin reduced csIL-1 α (**Fig 4A**) and transferred

active IL-1 α into the media (**Fig 4B,C**), suggesting pro-IL-1 α constitutes some of the csIL-1 α . Because all the IL-1 α antibodies used are raised against the mature IL-1 α C-terminus, pro-IL-1 α must tether via its N-terminus - otherwise thrombin cleavage would leave the antigenic C-terminus on the cell surface and the released fragment would be undetectable.

~20% of membrane proteins are GPI-anchored and can be specifically cleaved off with phosphoinositide phospholipase C (PI-PLC)[26]. Thus, incubation of EL4 cells with PI-PLC removed the prototypic GPI-anchored protein Thy-1 from the surface (**Fig 4D**), while the non-GPI-anchored CD45 or CD115 were not (**Fig 4E,F**), demonstrating specificity. Incubation of LPS-treated BMDMs with PI-PLC reduced csIL-1 α (**Fig 4G**), with denatured enzyme and vehicle controls without effect, and transferred cleaved IL-1 α to the media (**Fig 4H**). Furthermore, incubation of LPS-treated *Il1r2*^{-/-} BMDMs with PI-PLC reduced csIL-1 α to baseline (**Fig 4I**), suggesting loss of all csIL-1 α . However, as pro-IL-1 α doesn't contain signal sequences for translocation into the ER where GPI-modifications occur, this implies that csIL-1 α can interact with another GPI-anchored protein. Interestingly, csIL-1 α is reportedly tethered via a glycoprotein, which are usually GPI anchored[20], while GPI anchors require mannose residues and excess mannose disrupts csIL-1 α [20]. However, BMDMs treated with mannose (90 mg/ml as previously used[20]) led to cell permeabilisation that was potentiated by LPS (**Fig S3**), making determination of csIL-1 α impossible.

Interferon gamma inhibits translocation of IL-1 α to the cell surface. Macrophage polarisation dictates function, but if this effects csIL-1 α is not reported. M2 polarisation with IL-4/IL-13 before LPS treatment did not affect csIL-1 α level (**Fig 5A**). However, IFN γ treatment before LPS led to near complete loss of csIL-1 α (**Fig 5B**), with inhibition seen at incredibly low pg/ml concentrations (**Fig 5C**). In contrast, type-I interferons had no effect (**Fig 5D**). Due to this surprising sensitivity we tested an IFN γ neutralising antibody, which confirmed csIL-1 α inhibition was due to IFN γ activity (**Fig 5E**), and *Ifngr2*^{-/-} BMDMs, which verified csIL-1 α inhibition was via IFN γ receptor signalling (**Fig 5F**). Treatment with LPS first to establish csIL-1 α , followed by IFN γ did not alter csIL-1 α level (**Fig 5G**), suggesting IFN γ prevents csIL-1 α formation, rather than inducing shedding. IFN γ can sometimes inhibit IL-1 α / β expression[27]. However, IFN γ did not alter total IL-1 α expression within BMDMs by western blotting (**Fig 5H**) or intracellular cytokine staining (**Fig 5I**), or change expression of *Il1r2* (**Fig 5J**). Finally, although not a typical IFN γ source, BMDMs pretreated with IL-12 to induce (**Fig 5J**) or IL-10 to inhibit (**Fig 5K**) IFN γ didn't alter csIL-1 α , suggesting that macrophage-released IFN γ doesn't alter csIL-1 α expression. Together, this implies that IFN γ specifically prevents IL-1 α trafficking to the cell surface, rather than reducing global IL-1 α expression.

Senescent cells do not express cell surface IL-1 α , but do release IL-1 α . csIL-1 α is also reported to drive the SASP in human fibroblasts[4]. Thus, we evaluated growing and senescent human IMR-90 fibroblasts (a common model of senescence[6, 28]) for csIL-1 α . To validate the human anti-IL-1 α antibody we utilised siRNA knockdown in senescent cells, followed by intracellular cytokine staining for total IL-1 α . This showed ~90% less signal with IL-1 α -targeting siRNA compared to non-targeting control (**Fig 6A**), confirming antibody specificity. More csIL-1 α was found on senescent compared to growing cells (**Fig 6B**), in keeping with previous findings [4]. However, co-staining for cell permeability and putative csIL-1 α revealed no IL-1 α staining of viable senescent cells, but more IL-1 α in permeabilised cells (**Fig 6C**), and testing multiple cultures revealed no detectable csIL-1 α on senescent cells (**Fig 6D**). Indeed, deliberate permeabilisation revealed high IL-1 α expression in senescent cells, with much less in growing cells (**Fig 6E,F**), suggesting that inadvertent permeabilisation during csIL-1 α staining, combined with increased IL-1 α during senescence, would make senescent cells appear to express more csIL-1 α than growing cells. Because IL-1 α clearly drives the SASP[4, 5, 29] we tested conditioned media for soluble IL-1 α , which showed significant release (~120 pg/ml) from senescent cells, but none from growing cells (**Fig 6G**). Similarly, utilising an anti-IL-1 α ELISpot to capture IL-1 α as it is released from producing cells revealed multiple spots with senescent cells, and background levels in no-capture antibody controls (**Fig 6H**). Intriguingly, freshly harvested growing and senescent cells both showed similar low levels of permeabilisation (**Fig 6I**), suggesting that IL-1 α is unlikely to be released from senescent cells through

gasdermin D pores or permeabilised membranes. Finally, we also found no csIL-1 α on neutrophils, B-cells, T-cells and dendritic cells (DCs) (**Fig S5-8**), which have been varyingly described to express csIL-1 α [11, 21, 30-33].

DISCUSSION

Inflammation is a near-universal response that rapidly acts to reinstate homeostasis and remove the insult that perturbed the system. This innate immune response relies on speed to accomplish its objectives, with the specificity of adaptive immunity complementing the process and providing long-term host protection. However, if not carefully controlled, inflammation can result in greater damage to host tissues than the original insult. Thus, the immune system contains multiple levels of control and checkpoints to ensure responses are appropriately graded. Inflammasome activation is extensively controlled, given that activation leads to all-or-nothing release of IL-1 β and IL-18. However, as IL-1 α signals identically to IL-1 β and can be exteriorised independently of inflammasomes, IL-1 α may be a key mediator of initial low-level innate responses.

Using a robust validated system, we provide evidence that macrophages present genuine cell surface IL-1 α after activation. IL-1 α associates with the plasma membrane in part through its cognate receptor IL-1R2, and via association with a GPI-anchored protein. Importantly, trafficking of IL-1 α to the cell surface is exquisitely sensitive to IFN γ , which also induces MHC II on macrophages, and thus may prevent csIL-1 α expression during antigen presentation. In addition, these findings suggest that previous studies utilising IL-1 bioassays with fixed cells, or flow cytometry without a dead cell gate, likely detected intracellular IL-1 α , and thus multiple cell-types described to express csIL-1 α should be reappraised.

csIL-1 was reported before the discovery that IL-1 is the product of two distinct genes, and due to its induction by bacterial agents was proposed to be important in immune responses[14]. Most cytokines are secreted proteins, and thus can have far-reaching effects. In contrast, cell-associated cytokines can only induce autocrine or juxtacrine signalling, limiting effects to the immediate vicinity. This is clearly advantageous for powerful cytokines that induce cell death (e.g. TNF α) or activate both innate and adaptive immunity (e.g. IL-1), and thus potentially drive tissue damage and loss of tolerance. Many cell surface cytokines also exist as a soluble form, with shedding of membrane TNF α by ADAM17 the canonical route for secretion. However, both IL-1 α and IL-1 β can be released as soluble forms at high concentrations and induce identical signalling after ligating IL-1R1, begging the question, what does csIL-1 α do?

Macrophage IL-1 α/β release typically occurs after inflammasome engagement, which requires initial priming (e.g. LPS) to upregulate pro-IL-1 α/β and inflammasome components, followed closely by secondary signals (e.g. ATP) that activate the inflammasome. Thus, if a patrolling macrophage encounters a low-level infection, LPS would induce csIL-1 α and priming, but without significant cell death there would be no ATP to activate the inflammasome. This could enable a localised low-scale inflammatory response driven by csIL-1 α to try and eliminate infection with minimal tissue damage (e.g. ‘DEFCON 3’). If the insult generated host- or pathogen-derived proteases, csIL-1 α would be cleaved from the macrophage surface, allowing fully active IL-1 α to diffuse and signal more widely (e.g. ‘DEFCON 2’). If the infection grew, or the macrophage initially encountered an advanced infection causing host cell death, this would provide ATP (i.e. a danger signal) to activate the inflammasome, leading to high level IL-1 and IL-18 release and licensing of a full-scale immune response with accompanying collateral tissue damage (e.g. ‘DEFCON 1’).

csIL-1 α is reported to be the pro-IL-1 α form ([20, 22] and **Fig 4A-C**), and pro-IL-1 α is significantly less active than the mature form[6, 9, 10, 34]. Thus, the biological activity of csIL-1 α , *per se*, is unclear. Previous assays identified putative csIL-1 α via its activity, but we and others show that fixed macrophages used in these bioassays leak intracellular IL-1 α (**Fig 1B-D** and [17, 18]), and thus apportioning IL-1 α activity specifically to the cell surface form is technically challenging. Furthermore, any csIL-1 α associated via IL-1R2 (**Fig 3**) will be devoid of activity unless it dissociates and binds IL-1R1, which because of a higher affinity of IL-1 α for IL-1R1 (10^{-10} M) vs IL-1R2 (10^{-8} M) could make this plausible[35]. Alternatively, calpain[10], thrombin[11], caspase-5[6], granzyme B, elastase and chymase[9] all cleave pro-IL-1 α , resulting in increased activity and potential shedding of soluble mature

IL-1 α (as shown for thrombin in **Fig 4A-C** and [11]). Interestingly, most of these proteases could be considered to be activated under situations of ‘danger’[36] (e.g. calpain upon necrosis, thrombin during haemostasis, granzyme B during cytotoxic T-cell degranulation), and thus csIL-1 α on macrophages could be cleaved, resulting in activation and release to instigate signalling. Alternatively, given that IL-1 enhances T-cell expansion[37], if macrophage csIL-1 α has sufficient activity it could perhaps act as a form of co-stimulation during antigen presentation. However, as IFN γ induces macrophage MHC II expression[38], but profoundly inhibits macrophage csIL-1 α (**Fig 5**), this response could act to limit IL-1-driven Th17 cell differentiation and reinforce Th1 cells.

Most csIL-1 α studies utilised mouse macrophages (e.g. peritoneal, bone marrow-derived) or human monocytes. In addition, T-cells[30], B-cells[31], fibroblasts, endothelial[39] and DCs[21] are reported to express csIL-1 α . However, most studies used fixed cells with an IL-1 bioassay, or flow cytometry without a dead cell gate. In addition, pure DCs are incapable of releasing IL-1, with previous examples due to contaminating macrophages[40]. Using our validated system we show neither DCs, T-cells, B-cells nor neutrophils express true csIL-1 α before or after stimulation (**Fig S5-8**), suggesting previous findings may have been artefact, or the result of examining transformed lymphocyte clones[30, 31]. So why does csIL-1 α appear so unique to macrophages when multiple other cell types constitutively or inducibly express IL-1 α ? One possibility is that all macrophages we have tested do not express IL-1R1 (JNeC, MC unpublished), and thus macrophage csIL-1 α cannot bind its signalling receptor to induce autocrine stimulation, which could lead to sustained IL-1 signalling and potentially chronic inflammation. We were unable to disassociate csIL-1 α upon mannose treatment[20]. Instead, mannose lead to cell permeabilisation, and thus inability to distinguish csIL-1 α from intracellular IL-1 α (**Fig S3**). Thus, apparent csIL-1 α displacement by mannose[20], could be due to intracellular IL-1 α leakage from mannose permeabilised cells before fixation. We were also unable to detect csIL-1 α on senescent fibroblasts[4], but instead provide clear evidence that soluble IL-1 α is readily released (**Fig 6**).

Due to the limited knowledge on csIL-1 α , any role it may play in disease is not understood. csIL-1 α inhibits hepatocellular carcinoma development in a model using hepa1-6 cells stably expressing exogenous *Il1a* that had the nuclear localisation signal and calpain site mutated[41]. However, the outcome of these mutations was not sufficiently validated, and *Il1a* was not controlled by its endogenous promoter. Similarly, csIL-1 α worsens collagen destruction and arthritis in transgenic mice expressing truncated human IL-1 α from the chicken β -actin promoter[42]. However, transgenic cells released more soluble IL-1 α , making interpretation problematic. Thus, until we identify a unique mechanism for csIL-1 α tethering that we can modulate, the precise role of csIL-1 α remains unknown. We report IFN γ as the first negative regulator of IL-1 α trafficking to the cell membrane (**Fig 5**). However, we currently do not know the pathway downstream of IFN γ signalling that mediates this, or if in the future we could leverage this information to specifically inhibit csIL-1 α independent of other IFN γ effects (e.g. MHC II expression). However, IFN γ does not block csIL-1 α via reduced IL-1R2 expression (**Fig S4**). Similarly, we also don’t understand whether the csIL-1 α associated with IL-1R2 is a binding ‘artefact’ or represents an important biological pool of IL-1 α . Finally, we also show that IL-1 α release from senescent cells occurs independently of membrane pores or permeabilisation (**Fig 6I**), suggesting IL-1 α exits cells by an unknown mechanism, or is perhaps immediately cleaved and released from csIL-1 α as the substrate.

In conclusion, we show that *bone fide* csIL-1 α is expressed on the surface of macrophages after TLR ligation. csIL-1 α tethers to the plasma membrane through IL-1R2 or by association with a GPI-anchored protein, and can be cleaved, activated and released by proteases. Importantly, trafficking of pro-IL-1 α to the macrophage surface is inhibited by IFN γ . However, we find no evidence of csIL-1 α on other cell types. We believe this data is important for determining whether IL-1 α acts in a local or systemic context during immune responses, and thus how it contributes to disease and/or physiological processes.

MATERIALS AND METHODS

All materials from Sigma-Aldrich unless otherwise stated.

Cell culture

Male mice 8-12 weeks old on a C57BL/6J background were kept under UK Home Office authorisation at the University of Cambridge. *Il1a*^{-/-} mice were made by Horai[43]. *Il1r2*^{flox/flox} mice targeting exon 3 (kindly provided by Ari Waisman, University of Mainz, Germany; made by Taconic using standard homologous recombination), were crossed to B6.C-Tg(CMV-cre)1Cgn/J to generate *Il1r2*^{-/-} mice. Bone marrow was differentiated to macrophages in RPMI 1640 (Gibco), FCS (10%), L-glutamine (5 mg/ml), penicillin (10 U/ml), streptomycin (10 mg/ml), 2-mercaptoethanol (50 μM) and L929 cell-conditioned media (15%) for 6-7 d (37°C) in non-TC plates. For DCs, bone marrow was differentiated in full RPMI with GM-CSF (20 ng/ml; Peprotech) and incubated (7 d, 37°C). EL4 cells were cultured in full RPMI without L929. Murine fibroblasts were cultured in DMEM, FCS (10%), L-glutamine (5 mg/ml), penicillin (10 U/ml) and streptomycin (10 mg/ml). IMR-90 ER:*HRAS*^{G12V} cells, as previously described[28], were cultured in phenol-free DMEM (Invitrogen), FCS (10%), L-glutamine (5 mg/ml), penicillin (10 U/ml), streptomycin (10 mg/ml) and pyruvate (1%; Invitrogen). IMR-90 senescence was induced by culture (37°C; 7 d) with 4-hydroxytamoxifen (100 nM). T- and B-cells were obtained after squashing spleens through a cell strainer, with T-cells identified by CD3⁺ and B-cells by B220⁺. Blood neutrophils (EDTA) after RBC lysis (BioLegend) were identified by CD11b⁺ Ly6G⁺.

Cell treatment

BMDMs were plated, allowed to adhere (16 h, 37°C), and treated with LPS (1 μg/ml; Sigma), Pam3CSK4 (300 ng/ml), HKLM (10⁸ cells/ml), Poly(I:C) (10 μg/ml), FLA-ST (1 μg/ml), FSL-1 (100 ng/ml), ssRNA40 (5 μg/ml), ODN1826 (5 μM; all Invivogen) for 16 h at 37°C, or as stated. BMDMs ± LPS were also treated with emetine (1 nM), IL-4 (20 ng/ml), IL-13 (20 ng/ml), IFNγ (50 ng/ml, or as stated), IFNα (20 ng/ml, IL-12 (50 ng/ml), IL-10 (50 ng/ml; all Peprotech), D-mannose (90 mg/ml) or anti-IFNγ (5 μg/ml; BioLegend). Cells were detached with accutase (10 mins, RT) and either formaldehyde fixed (2%; 15 mins, RT; or as stated), or incubated with thrombin (2.5 - 10 U/ml in HBSS; 30 mins, RT; Merck Millipore), PI-PLC (0.05 - 0.55 U/ml in HBSS; 3 h, 37°C), heat denatured PI-PLC (5 mins, 100°C) or IL-1RA (0.5 - 1 μg/ml; 5 h, 37°C; Sobi), before conditioned media collection and/or csIL-1α staining. IMR-90 gene silencing utilised control or *IL1A*-targeted siRNA (12.5 nM; 24 h, 37°C; Dharmacon) delivered with RNAiMax (ThermoFisher).

Flow cytometry

For csIL-1α staining, cells were washed (PBS), Fc blocked (1:100; 10 mins, 4°C; Biolegend), stained with anti-IL-1α-PE (ALF-161; 1:20; 20 mins, RT; Biolegend) or anti-IL-1α-biotin (BL1a-89; 1:300; 20 mins, 4°C; Biolegend), and washed in flow buffer (1% BSA, 0.05% sodium azide in PBS). Biotinylated antibodies were detected with streptavidin-PE (1:1500; 20 mins, 4°C; eBioscience). Unless indicated samples were stained with Live/Dead (1:1000; 20 mins, 4°C; ThermoFisher). Propidium iodide staining (1 μg/ml; 5 mins, RT). Assessing human csIL-1α was as above, but used human Fc block (1:100, 10 mins, RT; Biolegend) and anti-IL-1α-FITC (FAB200F; 1:10; 20 mins, RT; R&D). csIL-1α detection in other cells included the lineage markers anti-Ly6G-PECy7 (1A8; 1:80; neutrophils), anti-B220-AF488 (RA3-6B2; 1:40; B-cells), or anti-CD3-FITC (17A2; 1:50; all Biolegend; T-cells). Cells staining also used anti-CD90-PE (30-H12; 1:80), anti-CD45-PECy7 (30-F11; 1:100), anti-CD11b-AF488 (M1/70; 1:800), anti-CD11c-AF488 (N418; 1:100; all 20 mins, RT; all Biolegend), anti-CD115-PE (AFS98; 1:100; 20 mins, RT; eBioscience). For total IL-1α staining, cells were formaldehyde fixed (2%; 15 mins, RT), washed, incubated with Permeabilisation Buffer (5 mins, RT; Biolegend), Fc blocked, stained with anti-IL-1α as above, washed in Permeabilisation Buffer, and resuspended in flow buffer. After final washing cells were analysed by flow cytometry (BD Accuri C6). Gating strategy is shown in **Fig 1E & S1**, with the csIL-1α +ve gate set so that unstained cells or stained cells without LPS or *Il1a*^{-/-} cells with LPS were not in it. Work followed flow cytometry guidelines[44].

IL-1 α bioassay

Conditioned media from thrombin-treated BMDMs was incubated with PPACK (10 nM; 15 mins, RT; Enzo). Primary murine fibroblasts were plated (12×10^3 /well, 48-well), allowed to adhere (16 h, 37°C) and treated with conditioned media (1:4 dilution) or IL-1 α (10 ng/ml; Peprotech), \pm anti-IL-1 α neutralising antibody (4 μ g/ml; R&D) for 5 h at 37°C. Supernatant was collected, clarified (400 g, 4 mins), stored (-20°C) and IL-6 concentration determined.

Cytokine quantification

Bead ELISAs (BD Biosciences) quantified mouse (IL-1 α , IL-6, TNF α) or human cytokines (IL-1 α), and were conducted following manufacturer's instructions. Briefly, sample and capture beads were incubated (1 h, RT) in 96-well filter plates (1.2 μ m; Merck Millipore), detection reagent added and incubation (1 h, RT). Bead washing (1% BSA in PBS) used a vacuum to drain wells. Beads analysis used flow cytometry and cytokine concentration determination used a standard curve. For IL-1 α ELISpot, PVDF plates (0.45 μ m; Millipore) were activated (35% EtOH; 30 mins, RT), washed (H₂O then PBS), coated with capture antibody (MAB200) or control (MAB4154; both 10 μ g/ml; 16 h, 4°C; R&D), washed (PBS), blocked (full DMEM; 2 h, RT), washed (PBS), cells plated and incubated (16 h, 37°C). Media was removed, adherent cells lysed (0.05% Tween 20/PBS; 10 mins, 4°C), wells washed (0.05% Tween 20/PBS), detection antibody added (BAF200; 0.2 μ g/ml; 2 h, RT; R&D), washed, and spots visualised with ELISpot Blue Color Module (SEL002; R&D).

Western blotting

BMDMs were lysed in RIPA buffer (ThermoFisher) with protease inhibitors, proteins (20 μ g) separated by SDS-PAGE, transferred to PVDF, blocked (5% milk powder/PBS; 1 h, RT), membranes incubated with anti-IL-1 α (AF-400-NA; 1:500; 16 h, 4°C; R&D) or anti-Tubulin (2148; 1:3333; 16 h, 4°C; CST), washed (0.05% Tween in PBS), incubated with anti-goat HRP (805-035-180; 1:2000; 1 h, RT; Jackson ImmunoResearch) and washed, before visualisation with ECL reagent (Amersham) and X-ray film (Fujifilm).

Gene expression analysis

RNA was isolated (RNeasy, Qiagen) and converted to cDNA using AMV reverse transcriptase (Promega). qPCR used Taqman probes with Amplitaq Gold (Life Technologies) in a RotorGene thermocycler (Corbett). Gene expression was evaluated using the $2^{-\Delta\Delta CT}$ method using *Gusb* and *Tbp* as reference genes.

Statistical Analysis

Statistical testing used Prism 8 (GraphPad). Unpaired t-tests compared two conditions. One-way ANOVA compared three or more conditions. If ANOVA was significant, Tukey's post-hoc compared all conditions to each other, and Dunnet's compared all conditions to control. Key assays were performed in duplicate.

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AUTHOR CONTRIBUTIONS

JNeC, MH, LK and DK performed and designed experiments, and analysed data. KF provided *Ifngr2^{-/-}* bones. MCHC conceived the project, designed experiments, analysed data, and wrote the manuscript with JNeC and MRB.

COMPETING INTERESTS

All authors declare no financial or commercial conflicting interests.

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FIGURE LEGENDS

Figure 1: Fixation or absence of a viability dye leads to misidentification of cell surface IL-1 α . (A) Flow cytometry reporting binding of an anti-IL-1 α antibody to putative cell surface IL-1 α (csIL-1 α) on mouse bone marrow-derived macrophages (BMDMs) treated \pm LPS, followed by fixation with formaldehyde as indicated (n=4 combined from four independent experiments). (B) ELISA for IL-1 α level in the conditioned media of BMDMs treated \pm LPS, followed by fixation with formaldehyde as indicated (n=5 combined from five independent experiments). (C,D) Flow cytometry showing cell permeability of J2 macrophages and BMDMs \pm fixation with 2% formaldehyde (n=2 combined from two independent experiments) (C), and permeability of BMDMs fixed with increasing concentrations of formaldehyde (n=3 combined from three independent experiments) (D). (E,F) Representative flow cytometry plots showing anti-IL-1 α staining of BMDM populations divided into a 'live' or 'dead' gates (representative of n=3)(E), or cell permeability of freshly harvested BMDMs (n=3 combined from three independent experiments) (F). Data represent mean \pm SEM; n value corresponds to independent experiments performed with one sample of BMDMs from different mice or cell lines per experiment on different days; p = ** \leq 0.01, **** \leq 0.0001, n.s. = not significant, using unpaired t-test (B,F) or 1-way ANOVA with Dunnett's (D).

Figure 2: LPS induces bona fide cell surface IL-1 α on macrophages. (A,B) Flow cytometry with ALF-161 anti-IL-1 α on wild-type (WT) and *Il1a*^{-/-} BMDMs treated \pm LPS for detection of putative csIL-1 α (n=4-6 combined from four to five independent experiments) (A), or treated \pm LPS and then permeabilised as indicated for detection of total IL-1 α (n=2 combined from two independent experiments) (B). (C) Flow cytometry with BL1a-89 anti-IL-1 α on WT and *Il1a*^{-/-} BMDMs treated with LPS and then permeabilised as indicated for detection of total IL-1 α (n=2 combined from two independent experiments). (D) Flow cytometry for csIL-1 α detected with BL1a-89 anti-IL-1 α on WT and *Il1a*^{-/-} BMDMs treated \pm LPS (n=3 combined from three independent experiments). (E-G) Flow cytometry for csIL-1 α detected with BL1a-89 anti-IL-1 α on WT BMDMs treated +LPS for the indicated time (n=3-6 combined from three to six independent experiments) (E) or \pm LPS for 16 h (n=19 combined from nineteen independent experiments) (F,G), or treated with a panel of Toll-like receptor ligands (n=2-7 combined from two to seven independent experiments) (H). (I-K) ELISA for TNF α level in the conditioned media (n=4 combined from four independent experiments) (I), western blot for IL-1 α with tubulin loading control (representative of n=2 independent experiments with one sample per experiment)(J) or flow cytometry for csIL-1 α detected with BL1a-89 anti-IL-1 α (n=4 combined from four independent experiments) (K) on WT BMDMs pre-treated \pm emetine before stimulation \pm LPS. Data represent mean \pm SEM; n value corresponds to independent experiments performed with one sample of BMDMs from different mice per experiment; p = * \leq 0.05, ** \leq 0.01, **** \leq 0.0001, n.s. = not significant, using unpaired t-test (A,D,F,I,K) or 1-way ANOVA with Tukey's (E) or Dunnett's (H).

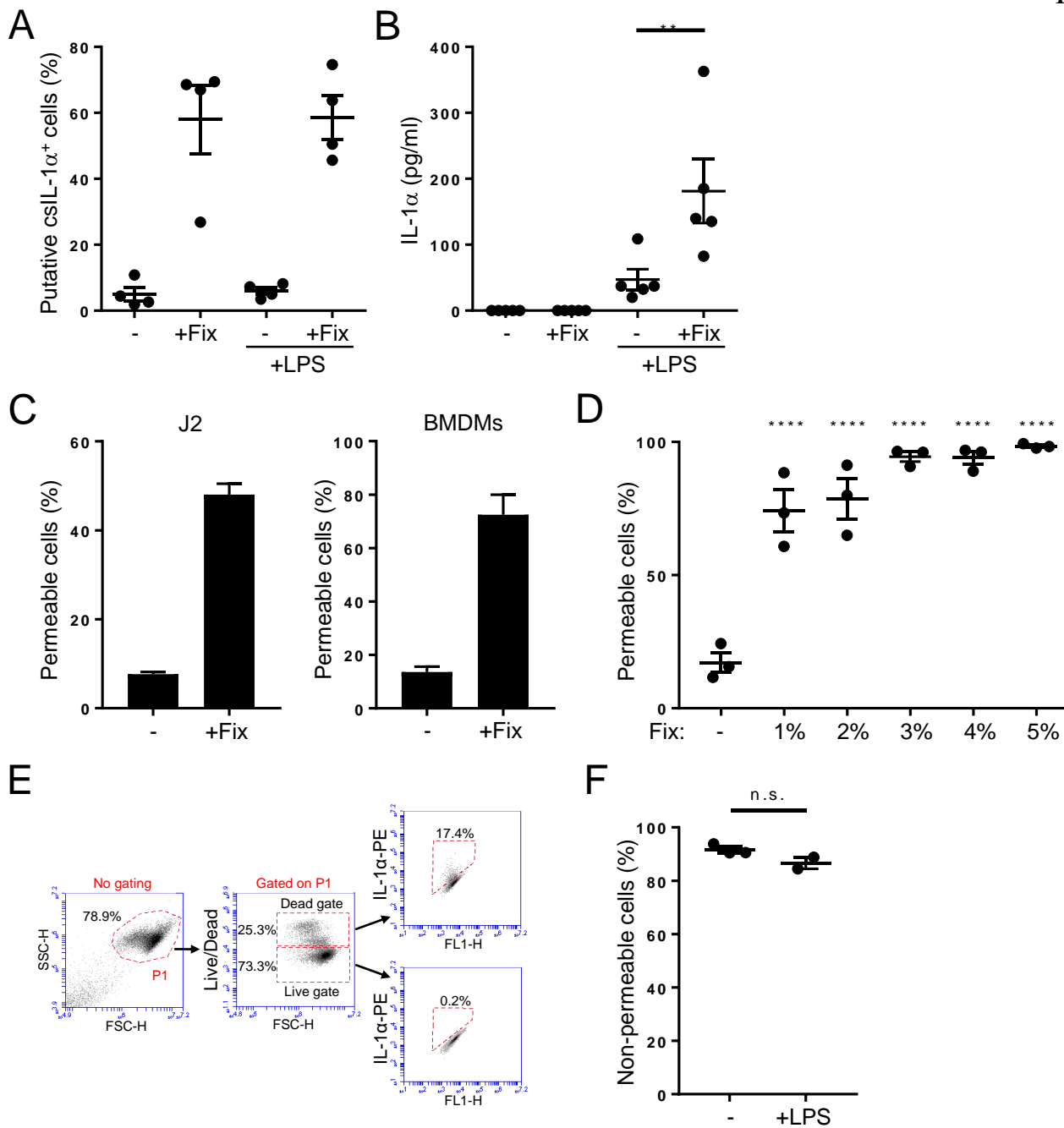
Figure 3: A proportion of IL-1 α associated with the cell membrane is dependent on the presence of IL-1 receptor 2. (A) Flow cytometry for csIL-1 α on wild-type (WT) BMDMs treated with LPS, followed by incubation with interleukin-1 receptor antagonist (IL-1RA) or IL-1 α before staining for csIL-1 α (n=5-10 combined from five to ten independent experiments). (B,C) Flow cytometry for csIL-1 α (n=7-9 combined from seven to nine independent experiments)(B) and western blot for total IL-1 α with tubulin loading control (representative of n=2 independent experiments with one sample per experiment) (C) on WT and *Il1r2*^{-/-} BMDMs treated \pm LPS. (D) Flow cytometry for csIL-1 α on *Il1r2*^{-/-} BMDMs treated with LPS, followed by incubation with IL-1RA or IL-1 α before staining for csIL-1 α (n=4 combined from four independent experiments). Data represent mean \pm SEM; n value corresponds to independent experiments performed with one sample of BMDMs from different mice per experiment; p = * \leq 0.05, n.s. = not significant, using unpaired t-test (B) or 1-way ANOVA (D) with Dunnett's (A).

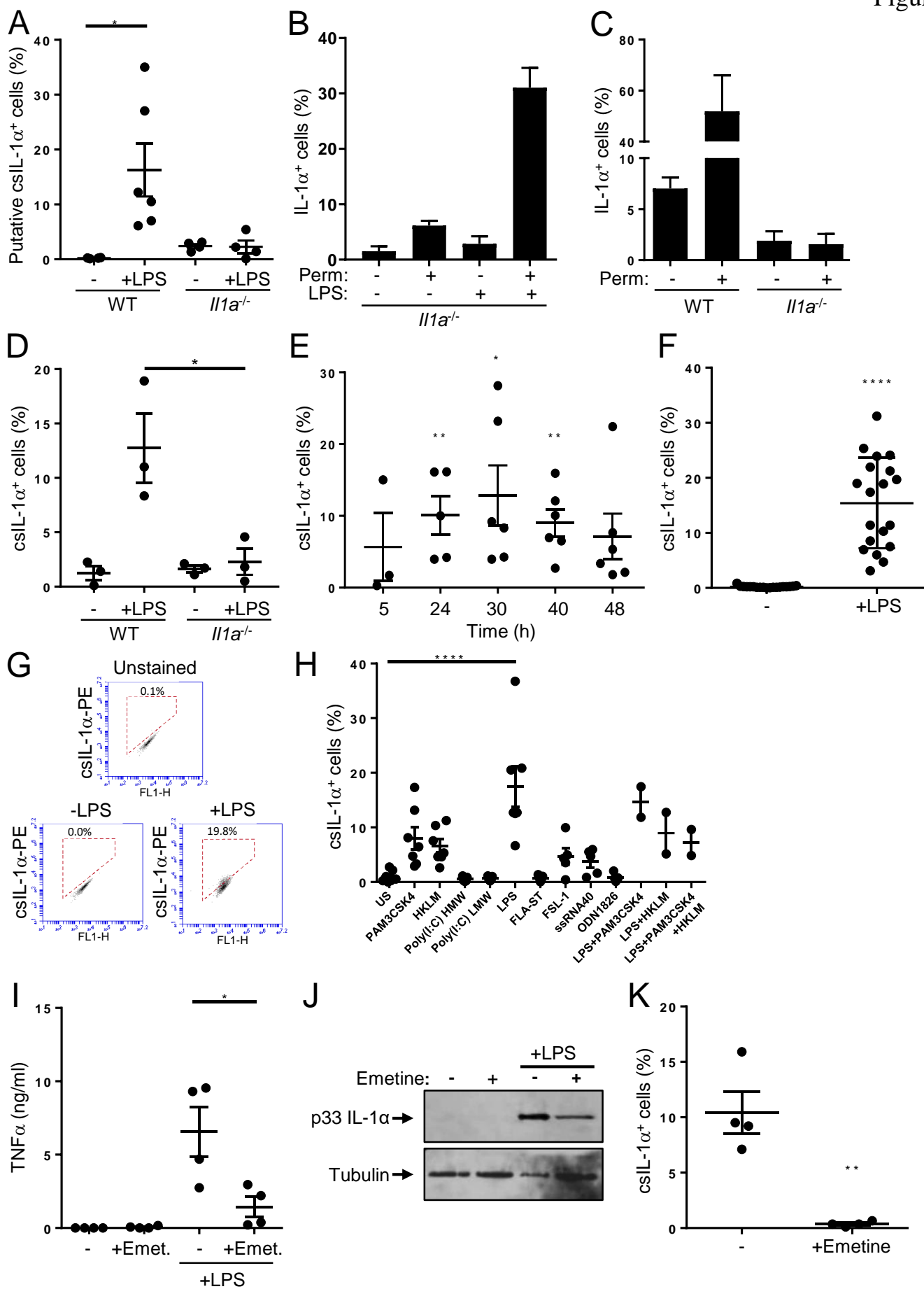
Figure 4: A proportion of cell surface IL-1 α associates with the membrane via a GPI-anchor. (A) Flow cytometry for csIL-1 α on BMDMs that were treated with LPS, followed by incubation \pm thrombin before staining for csIL-1 α (n=7 combined from seven independent experiments). (B) ELISA for IL-1 α

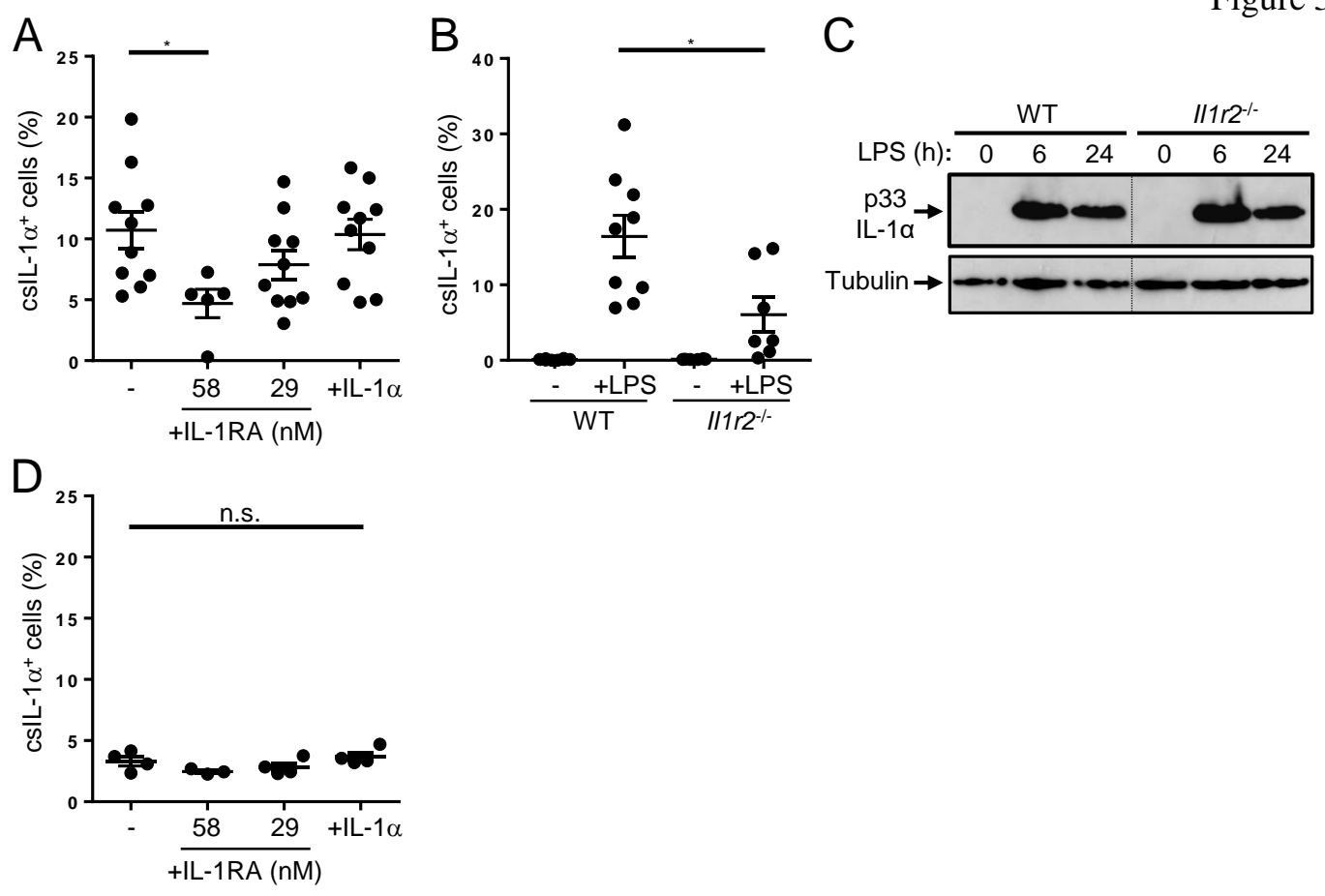
level in the conditioned media of BMDMs treated \pm LPS, followed by incubation \pm thrombin (10 or 2.5 U/ml) (n=6 combined from six independent experiments). (C) ELISA for IL-1-dependent IL-6 production by murine fibroblasts incubated \pm conditioned media from BMDMs (M ϕ CM) treated \pm LPS, followed by incubation \pm thrombin (Th), or IL-1 α , \pm an IL-1 α neutralising antibody (+ α Ab) (n=7 combined from seven independent experiments). (D-G) Flow cytometry for Thy-1 on EL4 cells (D), CD45 and CD115 on BMDMs (E,F) (D-F n=2 combined from two independent experiments) or csIL-1 α on LPS treated BMDMs (n=4-5 combined from four to five independent experiments) (G), after incubation with phosphoinositide phospholipase C (PI-PLC; 0.5 or 0.05 U/ml), heat denatured PI-PLC, or vehicle control before staining. (H) ELISA for IL-1 α level in the conditioned media of BMDMs treated with LPS, followed by incubation \pm PI-PLC (n=3 combined from three independent experiments). (I) Flow cytometry for csIL-1 α on *Il1r2*^{-/-} BMDMs treated with LPS, followed by incubation with PI-PLC before staining for csIL-1 α (n=3-4 combined from three to four independent experiments). Data represent mean \pm SEM; n value corresponds to independent experiments performed with one sample of BMDMs from different mice or cell lines per experiment, on different days; p = * \leq 0.05, ** \leq 0.01, *** \leq 0.001, **** \leq 0.0001, n.s. = not significant, using unpaired t-test (A,H) or 1-way ANOVA with Tukey's (B,C) or Dunnett's (G,I).

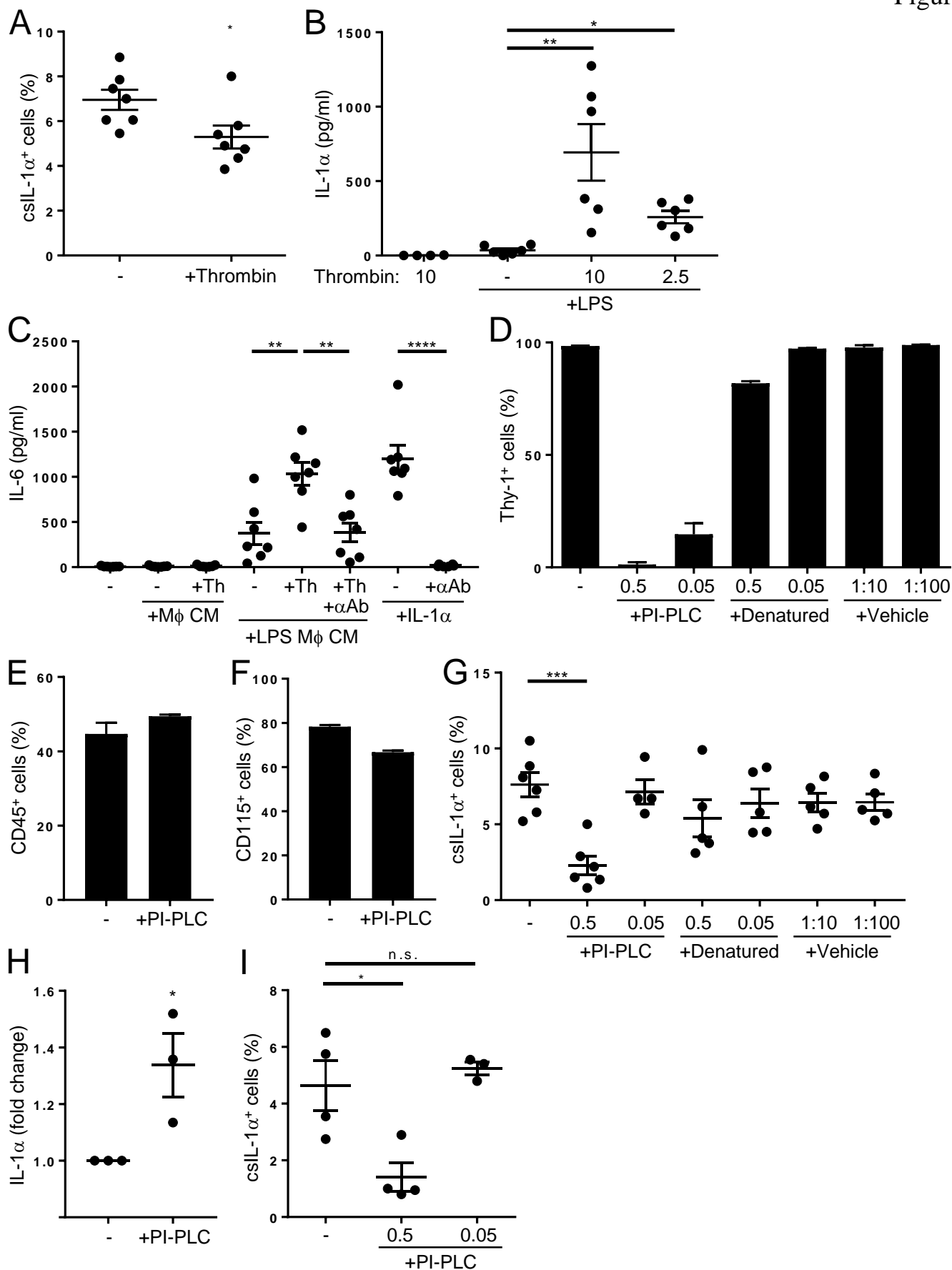
Figure 5: Low level interferon gamma inhibits trafficking of IL-1 α to the cell surface. (A-C) Flow cytometry for csIL-1 α on BMDMs that were treated \pm IL-4/IL-13 (A) or \pm IFN γ (B) (A,B n=2 combined from two independent experiments) before treatment \pm LPS, or treated with IFN γ at the concentrations indicated before LPS treatment (n=4-5 combined from four to five independent experiments)(C). (D,E) Flow cytometry for csIL-1 α on BMDMs that were treated \pm IFN α , \pm LPS (n=6 combined from six independent experiments) (D), or treated \pm IFN γ , \pm IFN γ neutralising antibody (+ γ Ab) and LPS (n=3 combined from three independent experiments) (E). (F) Flow cytometry for csIL-1 α on WT or *Ifngr2*^{-/-} BMDMs that were treated \pm IFN γ and \pm LPS (n=3-6 combined from three to six independent experiments). (G) Flow cytometry for csIL-1 α on BMDMs that were pretreated \pm LPS, followed by treatment \pm IFN γ (n=3 combined from three independent experiments). (H,I) Western blot with tubulin loading control (representative of n=2) (H) and intracellular cytokine staining by flow cytometry (n=2 combined from two independent experiments) (I) showing total IL-1 α in BMDMs that were treated \pm IFN γ and \pm LPS. (J,K) Flow cytometry for csIL-1 α on BMDMs that were pretreated \pm IL-12 (J) or \pm IL-10 (K) before treatment \pm LPS (I-K n=2 combined from two independent experiments). n value corresponds to independent experiments performed with one sample of BMDMs from different mice per experiment; p = * \leq 0.05, ** \leq 0.01, *** \leq 0.001, n.s. = not significant, using unpaired t-test (D,F,G) or 1-way ANOVA with Tukey's (E) or Dunnett's (C).

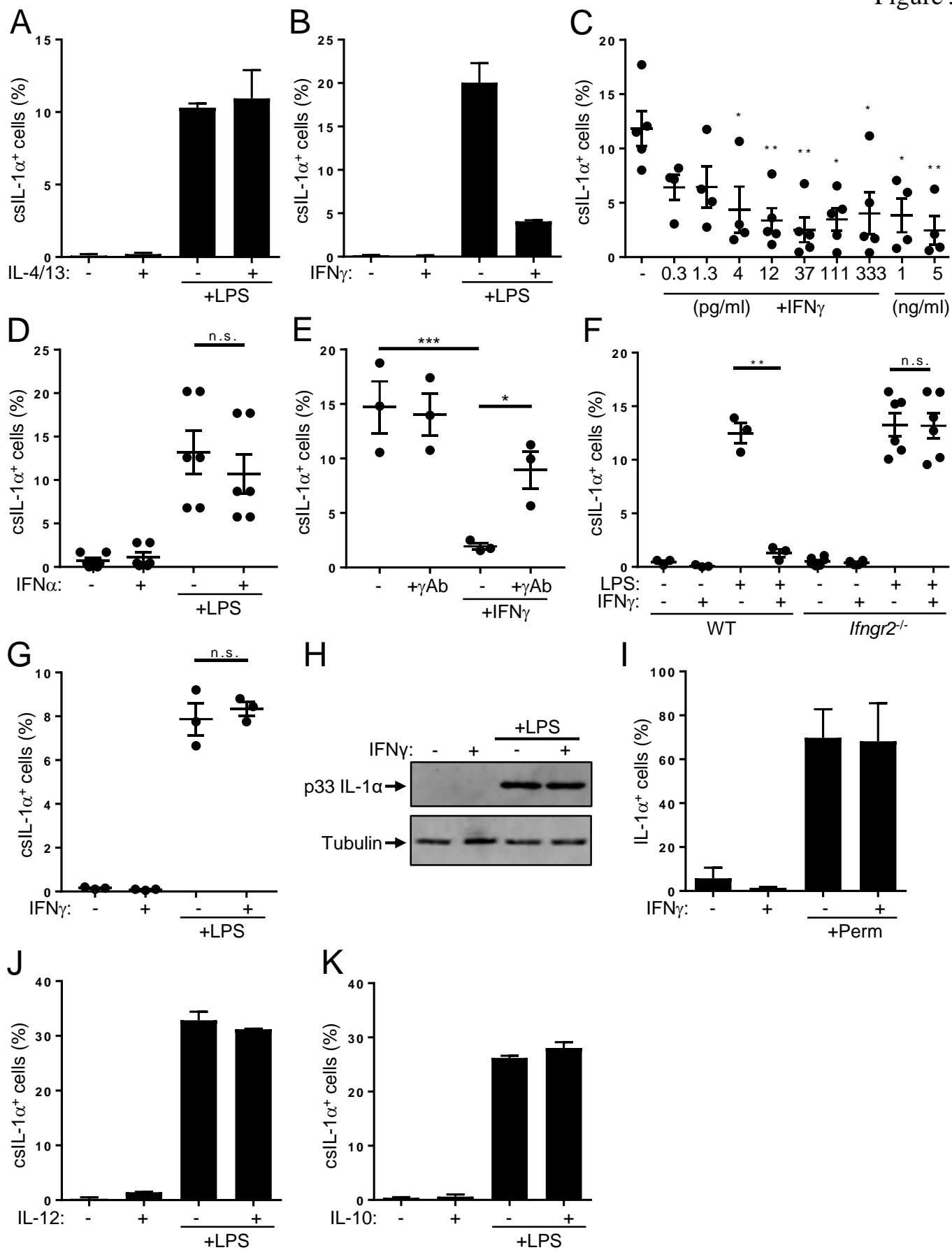
Figure 6: Senescent cells do not express cell surface IL-1 α . (A) Intracellular cytokine staining by flow cytometry showing total IL-1 α in senescent IMR-90 cells after transfection of non-targeted control (Cont) or *Il1a*-targeted siRNAs (n=3 combined from three independent experiments). (B) Representative flow cytometry for putative csIL-1 α on growing and senescent IMR-90 cells (representative of n=4 independent experiments with one sample per experiment). (C,D) Representative flow cytometry plots (C) and quantification (D) of csIL-1 α on senescent IMR-90 cell populations divided into 'live' or 'dead' gates (n=5 combined from five independent experiments). (E,F) Intracellular cytokine staining by flow cytometry showing total IL-1 α in growing and senescent IMR-90 cells (n=3 combined from three independent experiments). (G) ELISA for IL-1 α level in the conditioned media of growing (Grow) and senescent (Sen) IMR-90 cells (n=3 combined from three independent experiments). (H) Representative images showing spot forming cells using an IL-1 α ELISpot with senescent IMR-90 cells (representative of n=2). (I) Representative flow cytometry showing cell permeability of growing and senescent IMR-90 cells using Live/Dead or propidium iodide (PI) (representative of n=2 independent experiments with one sample per experiment). Data represent mean \pm SEM; n value corresponds to independent experiments performed on different days with one sample per experiment; p = * \leq 0.05, ** \leq 0.01, *** \leq 0.001, n.s. = not significant, using unpaired t-test (A,D,F,G).

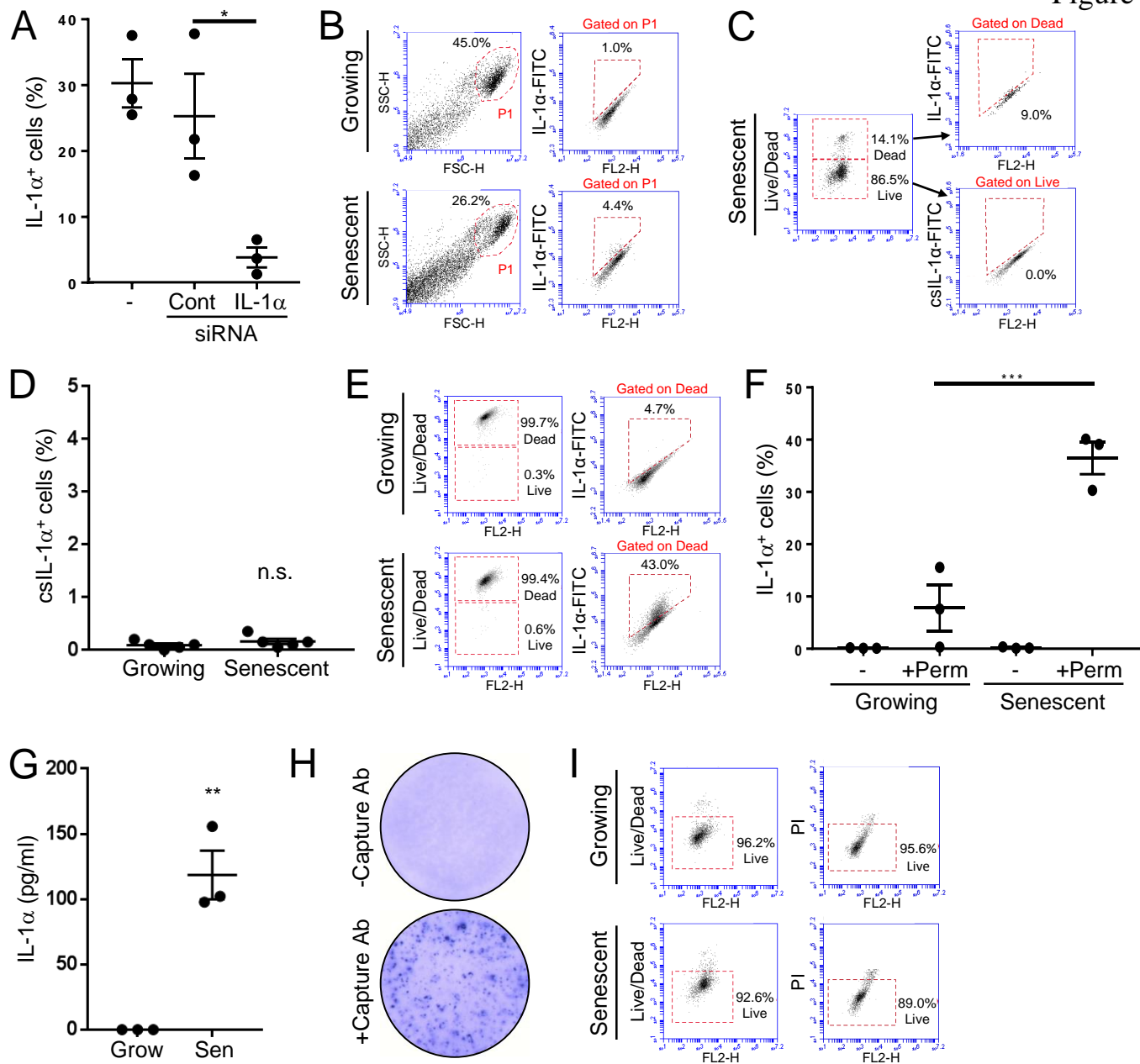












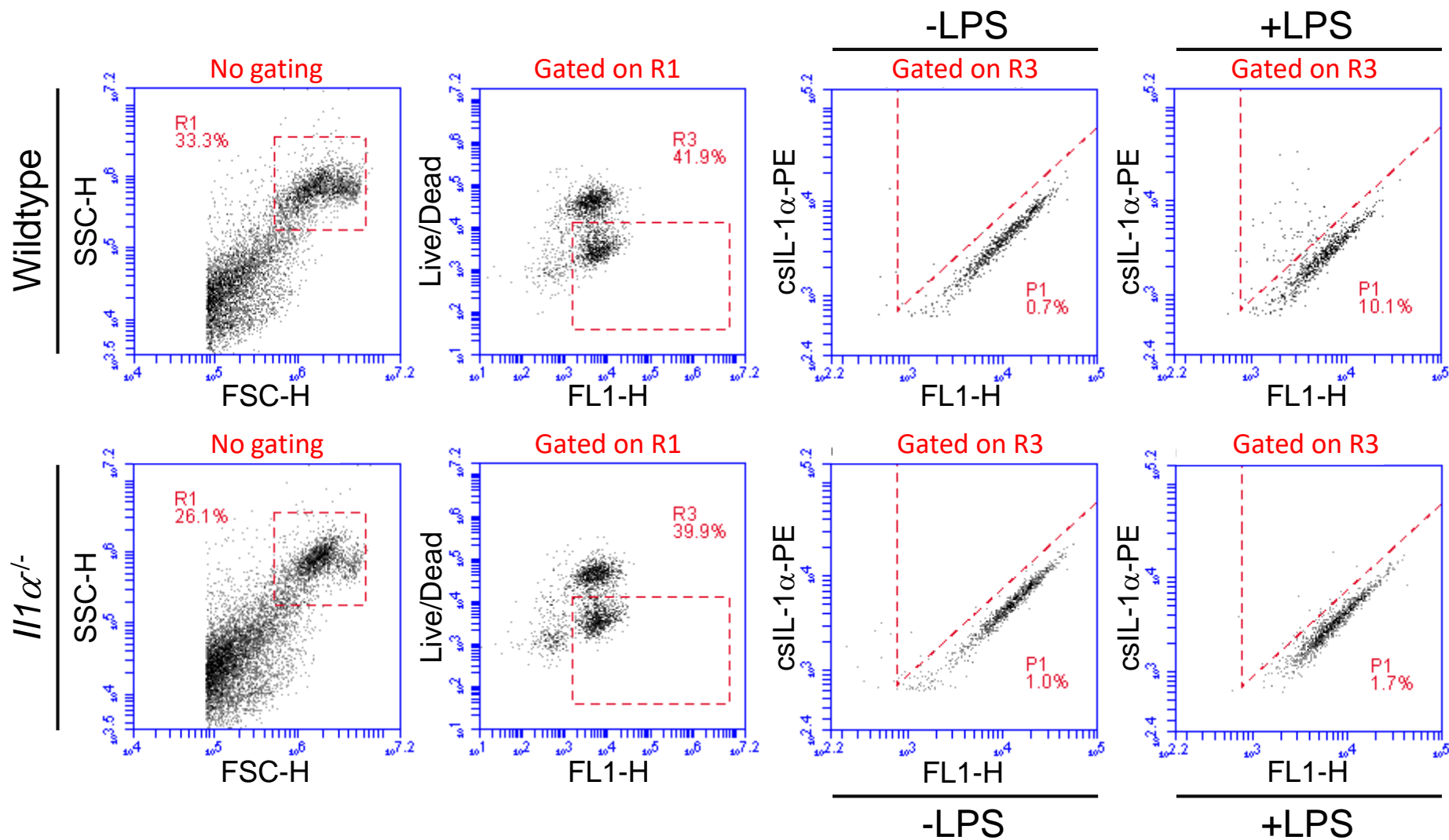


Figure S1: Macrophages from *Il1α*^{-/-} mice validates cell surface IL-1α detection. Representative flow cytometry plots for csIL-1α detected with BL1a-89 anti-IL-1α on wildtype and *Il1α*^{-/-} BMDMs treated ±LPS. BMDMs are gated by size, dead cells excluded and the csIL-1α positive gate set so that unstained cells, untreated stained cells or *Il1α*^{-/-} cells treated with LPS are not within it. Data representative of n=3 independent experiments (see Fig 2E).

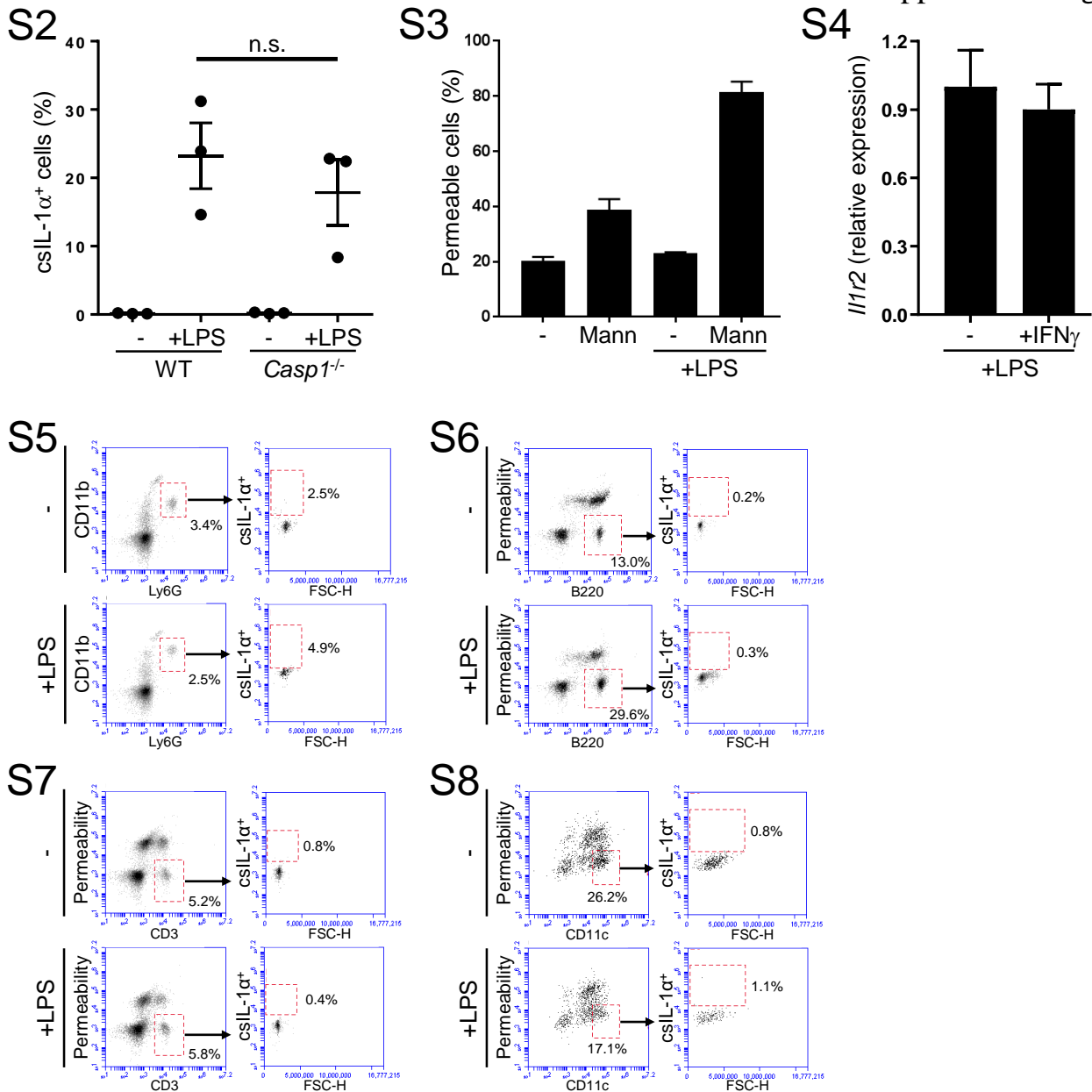


Figure S2: Expression of IL-1 α on the cell membrane is not dependent on caspase-1. Flow cytometry for csIL-1 α on wild-type (WT) and *Casp1*^{-/-} BMDMs treated \pm LPS. Data represent mean \pm SEM; n=3; n.s. = not significant.

Figure S3: IFN γ does not decrease *I11r2* expression. qPCR data showing relative expression of *I11r2* in LPS-treated BMDMs, \pm IFN γ treatment. Data represent mean \pm SEM; n=2.

Figure S4: Mannose treatment induces permeabilisation of macrophages. Flow cytometry showing cell permeability of BMDMs after treatment with D-mannose (Mann). Data represent mean \pm SEM; n=2.

Figure S5-8: Neutrophils, B cells, T cells or dendritic cells do not express cell surface IL-1 α . Flow cytometry plots showing csIL-1 α and lineage marker staining of neutrophils (S5), B cells (S6), T cells (S7) and dendritic cells (S8) treated \pm LPS. Data representative of n=2 (S5-8).