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Selective contribution of Tyk2 to cell activation by lipopolysaccharide

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Abstract **Tyk2 deficient mice show a markedly reduced susceptibility to lipopolysaccharide (LPS) induced shock and a partial impairment of IL-12 and interferon (IFN) signals. To examine the underlying mechanisms, we analysed the activation of peritoneal macrophages (PMΦs) and spleen cells after LPS challenge. In PMΦs and spleen cells the contribution of Tyk2 to the induction of the T-cell co-stimulatory molecules CD86, CD40 and MHC II was small or insignificant. By contrast, induced expression of the early activation marker CD69 on PMΦs and splenic cell populations required type I interferons (IFN-I) and Tyk2. The data suggest a selective contribution of Tyk2 to the activation of inflammation-relevant cell types by LPS.**

Keywords: CD69; CD86; Co-stimulatory molecule; Interferon (IFN); Janus kinase (Jak)

1. Introduction

Lipopolysaccharide (LPS) is a component of the outer membrane of Gram-negative bacteria and functions as an essential pattern for the recognition of invading pathogens by the host. Mammalian cells bind LPS through the Toll-like receptor (TLR) 4 signalling complex, which activates cells of the innate and adaptive immune system to engage in anti-bacterial defence [1]. Upon extensive or systemic exposure to LPS the host reacts with systemic inflammation and septic shock mediated through dysregulated pro-inflammatory cytokines and mediators [2]. The essential role of the LPS–TLR4 signalling cascade in the LPS-induced septic shock syndrome was emphasized by studies in mice carrying spontaneous, induced or targeted mutations in genes contributing to the pathway [3–5]. Disruption of the TLR4 signalling complex causes hypo-responsiveness to LPS and enhanced resistance to the endotoxin shock. Tumor necrosis factor alpha (TNFα) is an important mediator of the LPS-endotoxicity [6]. In addition, interferons (IFNs) and their signal transducers determine the sensitivity to LPS [7]. For example, mice deficient for IFNβ, IFN-γ-receptor, signal transducer and activator of transcription (Stat) 1, 4 or the tyrosine kinase 2 (Tyk2) show various degrees of LPS resistance [8–10]. Tyk2, a member of the Janus kinase (Jak) family, contributes to signal transduction by various cytokine receptors including those for IFN-I, interleukin (IL)-6, IL-10, IL-12 and IL-23. In previous studies we described an impairment of cytokine production/responses in LPS-treated, Tyk2 deficient mice and cells, resulting in endotoxin hyposensitivity [8,11]. Macrophages (MΦs), hematopoietic precursors and spleen cells are of primary importance for the recognition of LPS and/or the emerging and development of LPS toxicity [2]. Here we examined the activation of peritoneal macrophages (PMΦs) and spleen cells by LPS in absence of Tyk2 or the IFN-I receptor.

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Abbreviations: LPS, lipopolysaccharide; TLR, Toll-like receptor; Tyk2, tyrosine kinase 2; Ifnar, interferon type I receptor; IFN, interferon; IL, interleukin; PMΦs, peritoneal macrophages; DC, dendritic cell; NK cell, natural killer cell; MΦs, macrophages; FACS, fluorescence activated cell sorting

2. Materials and methods

2.1. Mice

Eight to twelve-week-old male C57BL/6 mice were purchased from Charles River Laboratories. Age- and sex-matched homozygous mutants of the lines B6.129P2-Tyk2^{tm1} (Tyk2−/−) [12], B6.129P2-Ifnar1^{tm1} (Ifnar1−/−) [13], B6.129P2-Ifnar^{tm1} (Ifnar2−/−) [14] and B6.129P2-Ifnb^{tm1} (IFNβ−/−) [15] were bred and housed in the specific pathogen free facility of Biomodels Austria according to FELASA guidelines. All animal experiments were discussed and approved by the institutional ethics committee and were carried out in accordance with protocols approved by the Austrian Laws (GZ 68.205/67-BrGT/2003; GZ 68.205/0204-C/GT/2007) and European Directives.

2.2. Preparation and culture of peritoneal macrophages (PMΦs)

Thioglycolate (Sigma–Aldrich)-elicited PMΦs were harvested as previously described [16]. Cells were cultured under standard conditions (37 °C, 5% CO_2, humidified atmosphere) in six well non-cell culture dishes (Greiner) for fluorescence activated cell sorting (FACS) analysis and six well cell culture dishes (BD Falcon) for RNA analysis, respectively, by using Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) supplemented with 5% fetal calf serum (FCS), 100 mg/ml penicillin, 100 U/ml streptomycin, 2 mM L-glutamine and 50 μM β-mercaptoethanol. Cells were stimulated either with 100 ng/ml LPS (derived from *Escherichia coli* serotype 055:B5) (Sigma–Aldrich) according to previously published data [8,10] or 100 U/ml IFNβ (Calbiochem) at indicated time points. For FACS analysis, MΦs were detached by incubating in ice cold phosphate-buffered saline (PBS)/15 mM EDTA at 4 °C, collected by gently pipetting up and down and resuspended in Hank's Buffered Salt Solution (HBSS)/25 mM HEPES, 5 mM $MgCl_2$, 0.1% BSA.

2.3. Preparation of spleen cells

Prior to cell isolation mice were injected intraperitoneally with LPS (*Escherichia coli* serotype 055:B5; 50 mg/kg body weight) (Sigma–Aldrich) according to previously published survival studies [8,10]. After

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dissecting the spleen, single cell suspension was prepared by mashing the tissue through a cell strainer (BD Falcon) subsequent to an enzymatic digestion with Collagenase D (0.5 mg/ml) and DNAseI (0.025 mg/ml) (Roche) in RPMI 1640 (Invitrogen). Cell fractions were isolated by density gradient using Lympholyte®-M (Cedarlane) according to manufacturer's protocol.

2.4. Flow cytometry

To minimize non-specific binding, cells were pre-incubated with anti-CD16/32 (Fcγ III/II Receptor) and 5% aggregated rat serum. For multicolour surface phenotyping 2.5×10^5 PMΦs and up to 1×10^6 spleen cells per reaction were incubated with following monoclonal antibodies: anti-CD4, anti-CD8a, anti-CD11b (Mac-1), anti-CD11c, anti-CD19, anti-CD40, anti-CD45R/B220, anti-CD69, anti-CD86 (B7-2), anti-I-A/I-E (MHC II), and anti-NK1.1, directly conjugated to fluorochromes (fluorescein isothiocyanate (FITC), phycoerythrin (PE), allophycocyanin (APC), Pacific Blue®, Alexa Fluor® 488, peridinin-chlorophyll-protein complex (PerCP)-Cy5.5 or PE-Cy7) all purchased from either BD Biosciences or BioLegend. The following lineage markers were used to distinguish defined cell populations: CD4 – helper T-cells, CD8 – cytotoxic T-cells, B220 – B-cells, NK1.1 – natural killer (NK) cells, CD11c/MHC II – mature dendritic cells (DCs) and CD11b – MΦs. For analysis at least 20 000 cells were acquired using a BD FACSAria Flow Cytometer (BD Biosciences) and data were analysed with FACSDiva™ 5.0.2 (BD Biosciences).

2.5. Preparation of RNA and quantitative real-time PCR (qPCR)

Total RNA was isolated with TRIzol® Reagent (Invitrogen). RNA was treated with RQ1 RNase-Free DNase (Promega) and reverse transcribed using iScript™ cDNA synthesis kit (Biorad). All protocols were performed following the manufacturer's recommendations. For TaqMan® real-time PCR, a standard reaction contained 2 µl cDNA, 4 mM $MgCl_2$, 300 nM primers (Invitrogen), 100 nM probe (Metabion), 1U HOT FIREPol®, 1× PCR reaction buffer B (Solis BioDyne), 200 µM dNTP mix. Amplification was performed by an initial denaturation step at 95 °C for 15 min followed by 45 cycles at 95 °C for 30 s and 60 °C for 1 min on an ABI PRISM 7700 (Applied Biosystems). For the detection of expression of CD69, primers and probe from a previously published assay were used [17]. Sample values were normalized to the expression of the housekeeping gene ubiquitin-conjugating enzyme E2D2 (UBE2D2) using the primers: UBE2D2forward 5′-AGG TCC TGT TGG AGA TGA TAT GTT-3′, UBE2D2reverse 5′-TTG GGA AAT GAA TTG TCA AGA AA-3′ and the probe FAM-CCA AAT GAC AGC CCC TAT CAG GGT GG-BHQ-1.

2.6. Statistical analysis

Results are presented as means ± S.E.M. and are representative of at least three independent experiments. Differences between groups were determined by univariate ANOVA analysis using SPSS for Windows 14.0 software. A *P*-value less than 0.05 denoted the presence of a statistically significant difference with the following degrees: $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

3. Results and discussion

3.1. Expression of CD69 in LPS-treated PMΦs requires Tyk2 and signalling through the IFN-I receptor

To study the immediate response of Tyk2 deficient PMΦs the cells were stimulated with LPS over a period of 24 h and the expression of the early activation cell surface marker CD69 was measured by FACS analysis. CD69 is a member of the C-type lectin family and is rapidly upregulated by various stimuli, including IFN-I, in most cells of the innate and acquired immune system [17,18]. An increase of $CD69^+$ cells was evident in wild-type PMΦs 6 h after stimulation and peak values were reached at 12 h with approximately 50% positive cells (Fig. 1A). Beyond 12 h stimulation $CD69^+$ PMΦs rapidly declined. The numbers of untreated $CD69^+$ Tyk2−/− PMΦs were comparable to wild-type cells, however, reduced numbers of $CD69^+$ Tyk2 deficient cells were observed after LPS stimulation. Upregulation of CD69 in LPS-treated PMΦs deficient for Ifnar1, Ifnar2 and IFNβ was almost completely abolished (Fig. 1A and B). Stimulation of PMΦs with IFNβ alone revealed that up-regulation of $CD69^+$ cells was partially dependent on Tyk2 and, as expected, completely dependent on Ifnar1 (Fig. 1C). The expression of CD69 mRNA upon LPS

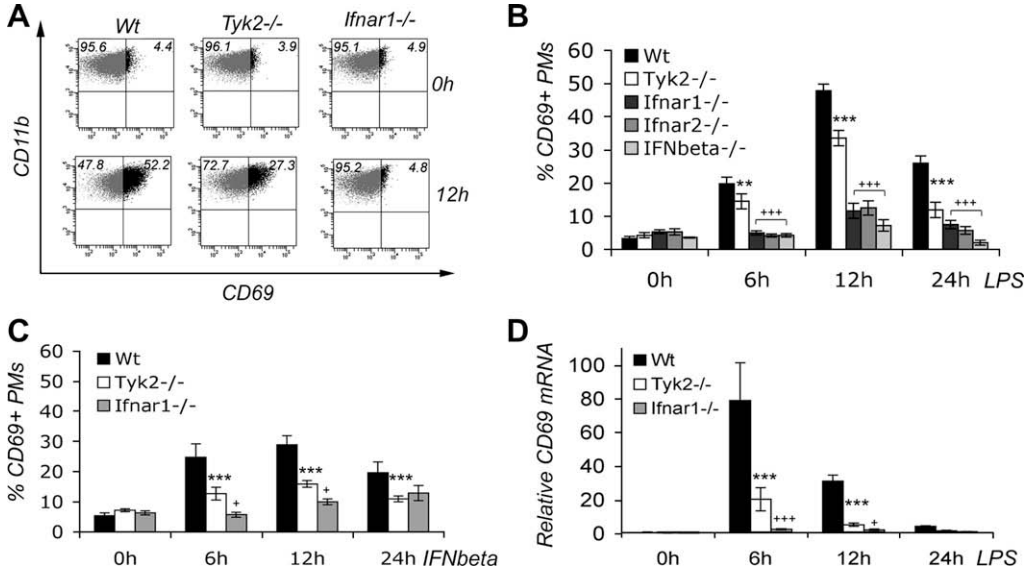

Fig. 1. (A–C) FACS analyses of CD69 expression on peritoneal macrophages, PMΦs (PMs). (A) Dot plot analysis of $CD11b^+$ gated PMs. The percentage of $CD69^+$ cells is given in the plot panels. Untreated (0 h) and LPS (100 ng/ml) stimulated cells (12h) are shown. (B, C) Time-course of the induction of $CD69^+$ cells in the respective genotypes following stimulation with LPS (100 ng/ml) and IFNβ (100 U/ml), respectively. (D) Real-time analysis of relative CD69 mRNA expression in PMs upon LPS stimulation (100 ng/ml). Values were calibrated to Wt 0 h. Data are presented as means ± S.E.M. and represent at least three independent experiments. For each experiment at least two pools (two-three animals per pool) per genotype were analysed. Significance is indicated as: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$; $^{*}P$ Tyk2−/− compared to Wt, ^{+}P Ifnar1−/− compared to Tyk2−/−.

treatment was clearly diminished in Tyk2−/− PMΦs and nearly absent in Ifnar1−/− PMΦs suggesting impaired transcription as a major cause of the reduced cell surface expression of CD69 in the mutant genotypes (Fig. 1D). Nevertheless, post-transcriptional modifications contributing to the aberrant mutant cell surface expression patterns cannot be excluded. Taken together, IFNβ and interferon type I receptor (Ifnar) are both essential for the increased expression of CD69 in PMΦs upon LPS treatment. Absence of Tyk2 causes a partial reduction of the LPS/IFN-I effect on CD69 expression in accordance with the previously described partial requirement of Tyk2 for IFN-I responses [12,19].

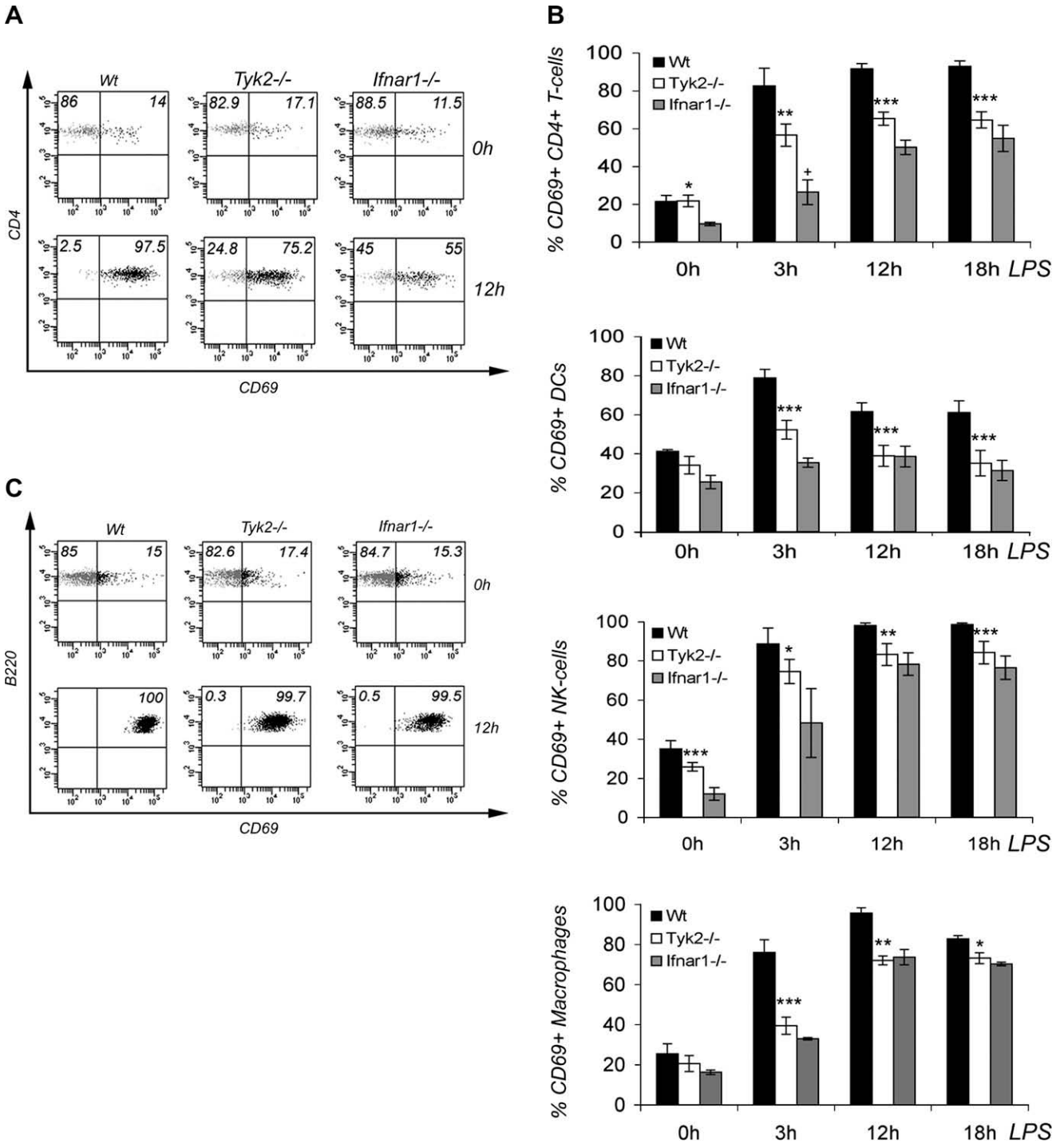

Fig. 2. FACS analyses of CD69 expression on spleen cells after LPS challenge (50 mg/kg). (A) Dot plot analysis of spleen cells gated for $CD4^+$ T-cells. The percentage of $CD4^+CD69^+$ T-cells is given in the plot panels. (B) Time-course of the induction of $CD69^+$ cells gated on $CD4^+$ T-cells, $CD11c^+$MHC II^+ DCs, $NK1.1^+$ NK-cells and $CD11b^+$ macrophages at indicated time-points for the respective genotypes: Wt, black columns; Tyk2−/−, white columns; Ifnar1−/−, grey columns. (C) Dot plot analysis of spleen cells gated for $B220^+$ B-cells. The percentage of $B220^+CD69^+$ B-cells is given in the plot panels. Cells of untreated (0 h) and LPS treated mice at the indicated time-points are shown. Results are presented from at least three independent experiments, each with two mice per time-point and genotype. For statistical analysis see legend of Fig. 1.

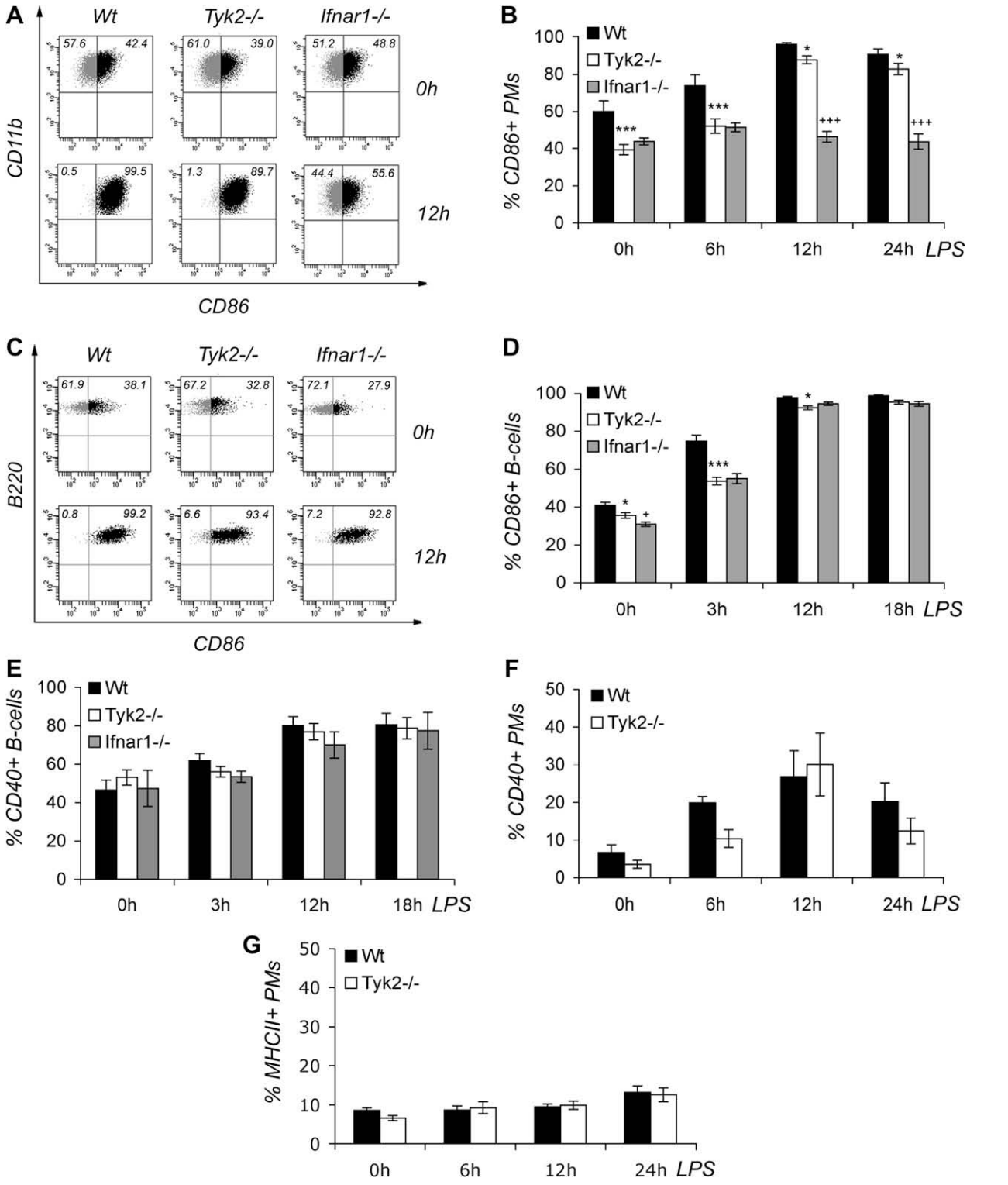

Fig. 3. (A–D) FACS analyses of CD86 expression. (A, C) Dot plot analyses of CD11b$^+$ gated peritoneal macrophages, PMΦs (PMs) and B220$^+$ gated B-cells. Cells of untreated (0 h) and LPS (50 mg/kg) treated mice (12 h) are shown. The percentage of CD11b$^+$CD86$^+$ PMs and B220$^+$CD86$^+$ B-cells respectively is given in the plot panels. (B, D) Time-course of the induction of CD86$^+$ cells gated on CD11b$^+$ PMs and B220$^+$ B-cells respectively at indicated time-points for the respective genotypes: Wt, black columns; Tyk2−/−, white columns; Ifnar1−/−, grey columns. (E, F) Time-course of the induction of CD40$^+$ cells gated on (E) B220$^+$ B-cells and (F) CD11b$^+$ PMs, respectively. (G) Time-course of the induction of MHC II$^+$ cells gated on CD11b$^+$ PMs. For experimental procedures and statistical analysis see legends of Fig. 1 and Fig. 2.

3.2. Cell type-specific regulation of CD69 expression in the spleen of LPS-treated mice

Next we analysed spleen cells of LPS-treated mice for early activation markers. Spleen-derived T-cells, DCs, NK cells and MΦs showed an increase of $CD69^+$ cells in all genotypes. With the exception of B-cells, however, all cell types from Tyk2−/− and Ifnar1−/− spleens showed a clear reduction in the number of $CD69^+$ cells throughout the whole time-course of stimulation (Fig. 2A and B). Significant differences between the numbers of $CD69^+$ Tyk2−/− and Ifnar1−/− cells were found only at the earliest time-point after treatment, with T-cells from Ifnar1−/− animals expressing lower levels of CD69 (Fig. 2B). The difference between Tyk2−/− and wild-type was most evident for $CD4^+$ and $CD8^+$ T-cells, DCs and MΦs and less pronounced for NK cells (Fig. 2A and B and data not shown). In B-cells the increase of $CD69^+$ cells was delayed in Tyk2−/− and Ifnar1−/−, but reached wild-type levels at 12 h post-stimulation (Fig. 2C and data not shown).

Contrasting PMΦs, Tyk2 and Ifnar1 deficiency had similar and partial effects on LPS-induced CD69 expression in all spleen cell populations with the exception of B-cells. The increase of $CD69^+$ cells in the absence of Ifnar1 can be explained by co-stimulatory factors and cellular cross-talk in whole organs and/or co-culture conditions. Consistent with this notion, upregulation of CD69 by LPS in T-cells is stimulated by co-culture with MΦs in a complement receptor dependent manner [20]. The early activation of B-cells is unaffected by the lack of IFN-I signalling. Instead, direct interaction with T-cells and the presence of an additional B-cell specific LPS receptor (RP105/CD180) are responsible for the LPS-induced increase in CD69 expression in this cell type [21,22].

3.3. Involvement of Tyk2 in LPS-mediated upregulation of CD86 on PMΦs and B-cells

The initiation of the adaptive immune response depends on co-stimulatory surface molecules such as CD86 (B7-2) and CD40, which are expressed by antigen presenting cells and help to activate T-cells that are exposed to antigen in the context of major histocompatibility complex (MHC) proteins. Consistent with previous reports showing that upregulation of CD86 in LPS-treated PMΦs requires TRIF mediated IFN-I synthesis and signalling, CD86 expression on these cells was reduced in absence of Ifnar1 ([23] and Fig. 3A and B). Numbers of $CD86^+$ PMΦs were reduced in absence of Tyk2 or Ifnar1 both in untreated cells and in cells after treatment with LPS for 6 h. At later time points, however, Tyk2−/− PMΦs nearly matched the wild-type situation indicating that the participation of Tyk2 to the IFN-I response is less crucial for the increase in LPS-mediated CD86 expression (Fig. 3A and B). Neither Ifnar1 (see also [24]) nor Tyk2 were required for the LPS-induced increase of $CD86^+$ B-cells (Fig. 3C and D), which is in accordance with the demonstration of an Ifnar-independent alternate pathway of B-cell activation by LPS [21,22].

Upregulation of CD40 and MHC II in PMΦs and B-cells was completely independent of Tyk2 (Fig. 3E–G). This is in line with previous findings that T-cell co-stimulatory molecule induction in spleen cells by various stimuli is widely unaffected by defects in IFN-I signals [19,24,25].

Taken together, we demonstrate a clear Tyk2 requirement for the expression of the early activation marker CD69 in LPS-treated PMΦs and, upon in vivo challenge, in splenic MΦs, T-cells, DCs and NK cells, but not in B-cells. In accordance with previous findings IFN-I are important inducers of CD69 cell surface expression. CD69 is associated with different NK cell activation pathways [26] and has an impact on T-cell proliferation and activation [27]. The role of CD69 in the endotoxin shock remains to be elucidated. Nevertheless, it seems conceivable that the diminished early immune cell activation via CD69 in Tyk2−/− mice contributes to the reduced cytokine production and response observed during the development of the LPS-induced septic shock.

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