Journal of Innate Immunity

Research Article

J Innate Immun 2024;16:226–247
DOI: 10.1159/000538282

Received: February 17, 2023
Accepted: March 6, 2024
Published online: March 25, 2024

Type I Interferon, Induced by Adenovirus or Adenoviral Vector Infection, Regulates the Cytokine Response to Lipopolysaccharide in a Macrophage Type-Specific Manner

Mareike D. Maler[a, b] Sophie Zwick[c] Carsten Kallfass[d] Peggy Engelhard[c] Hexin Shi[e] Laura Hellig[c] Pang Zhengyang[f] Annika Hardt[c] Gernot Zissel[c] Zsolt Ruzsics[d] Willi Jahnen-Dechent[g] Stefan F. Martin[b] Peter Jess Nielsen[a] Daiana Stolz[c] Justyna Lopatecka[h] Sarah Bastyans[h] Bruce Beutler[e] Wolfgang W. Schamel[f, i, j] György Fejer[a, h] Marina Alexandra Freudenberg[a, c, i]

[a]Max-Planck Institute of Immunobiology and Epigenetics, Freiburg, Germany; [b]Allergy Research Group, Department of Dermatology, Medical Center – University of Freiburg, Faculty of Medicine, Freiburg, Germany; [c]Department of Pneumology, Medical Center – University of Freiburg, Faculty of Medicine, Freiburg, Germany; [d]Institute of Virology, Medical Center – University of Freiburg, Faculty of Medicine, Freiburg, Germany; [e]Center for the Genetics of Host Defense, University of Texas Southwestern Medical Center, Dallas, TX, USA; [f]Faculty of Biology, University of Freiburg, Freiburg, Germany; [g]Signalling Research Centres BIOSS and CIBSS, University of Freiburg, Freiburg, Germany; [h]Helmholtz-Institute for Biomedical Engineering, RWTH Aachen University, Faculty of Medicine, Aachen, Germany; [i]Center of Chronic Immunodeficiency CCI, University Clinics and Medical Faculty, Freiburg, Germany; [j]School of Biomedical Sciences, Faculty of Health, University of Plymouth, Plymouth, UK

Keywords
Lipopolysaccharide · Adenoviral vector · Macrophages · IFN-αβ · Cytokines

Abstract
Introduction: While TLR ligands derived from microbial flora and pathogens are important activators of the innate immune system, a variety of factors such as intracellular bacteria, viruses, and parasites can induce a state of hyperreactivity, causing a dysregulated and potentially life-threatening cytokine over-response upon TLR ligand exposure. Type I interferon (IFN-αβ) is a central mediator in the induction of hypersensitivity and is strongly expressed in splenic conventional dendritic cells (cDC) and marginal zone macrophages (MZM) when mice are infected with adenovirus. This study investigates the ability of adenoviral infection to influence the activation state of the immune system and underlines the importance of considering this state when planning the treatment of patients. ***Methods:*** Infection with adenovirus-based vectors (Ad) or pretreatment with recombinant IFN-β was used as a model to study hypersensitivity to lipopolysaccharide (LPS) in mice, murine macrophages, and human blood samples. The TNF-α, IL-6, IFN-αβ, and IL-10 responses induced by LPS after pretreatment were measured. Mouse knockout models for MARCO, IFN-αβR, CD14, IRF3, and IRF7 were used to probe the mechanisms of the hypersensitive reaction. ***Results:*** We

karger@karger.com
www.karger.com/jin

Correspondence to:
György Fejer, gyorgy.fejer@plymouth.ac.uk
Marina Alexandra Freudenberg, marina.freudenberg@uniklinik-freiburg.de

show that, similar to TNF-α and IL-6 but not IL-10, the induction of IFN-αβ by LPS increases strongly after Ad infection. This is true both in mice and in human blood samples ex vivo, suggesting that the regulatory mechanisms seen in the mouse are also present in humans. In mice, the scavenger receptor MARCO on IFN-αβ-producing cDC and splenic marginal zone macrophages is important for Ad uptake and subsequent cytokine overproduction by LPS. Interestingly, not all IFN-αβ-pretreated macrophage types exposed to LPS exhibit an enhanced TNF-α and IL-6 response. Pretreated alveolar macrophages and alveolar macrophage-like murine cell lines (MPI cells) show enhanced responses, while bone marrow-derived and peritoneal macrophages show a weaker response. This correlates with the respective absence or presence of the anti-inflammatory IL-10 response in these different macrophage types. In contrast, Ad or IFN-β pretreatment enhances the subsequent induction of IFN-αβ in all macrophage types. IRF3 is dispensable for the LPS-induced IFN-αβ overproduction in infected MPI cells and partly dispensable in infected mice, while IRF7 is required. The expression of the LPS co-receptor CD14 is important but not absolutely required for the elicitation of a TNF-α over-response to LPS in Ad-infected mice. ***Conclusion:*** Viral infections or application of virus-based vaccines induces type I interferon and can tip the balance of the innate immune system in the direction of hyperreactivity to a subsequent exposure to TLR ligands. The adenoviral model presented here is one example of how multiple factors, both environmental and genetic, affect the physiological responses to pathogens. Being able to measure the current reactivity state of the immune system would have important benefits for infection-specific therapies and for the prevention of vaccination-elicited adverse effects.

Introduction

TLR ligands, as a part of the microbial flora and of pathogens, are ubiquitously present in the mammalian organism. Triggering of the innate immune system by such activators, including the TLR4 ligand bacterial lipopolysaccharide (endotoxin; LPS), and the subsequent pro-inflammatory cytokine response, are crucial for defense against invading pathogens [1, 2]. On the other hand, the reactivity of the innate immune system to LPS depends on environmental factors and is under constant fluctuation. Infections with a range of intracellular bacteria, viruses, and parasites may lead to innate immune hyperreactivity (hypersensitivity) [3–5]. Under these conditions, subsequent LPS exposure leads to a dysregulated cytokine over-response with damaging consequences, including vascular leakage, coagulopathy, organ dysfunction, and, in the worst-case scenario, shock and death [6]. Cytokine overproduction in the course of influenza or COVID-19 infection in humans is the cause of acute lung injury or the more severe acute respiratory distress syndrome [7].

Type I interferon (IFN-αβ) is the key mediator in the viral induction of hypersensitivity to LPS [4, 8]. IFN-αβ represents a family of several IFN-α and single IFN-β, -κ, -ω, and -ε members, all of which activate the same IFN-αβ receptor [9]. As shown in mice, IFN-αβ drives sensitization of the innate immune system during the early stages of viral infection, while in later stages, it does so in synergy with IL-18 *via* subsequent induction of type II interferon [10–12]. Mice infected with human adenoviruses (Ad) or Ad-based vectors represent a suitable model to study the early induction of LPS hypersensitivity by IFN-αβ alone. After infecting murine cells, adenoviral DNA directly induces IFN-αβ production *via* the cytoplasmic cGAS-STING pathway [13]. Viral DNA is translocated into the cell nucleus, and the early adenovirus-encoded genes are expressed. However, the viral genome is not replicated, and new viral particles are not made [14, 15].

Conventional dendritic cells (cDC) and macrophages in the spleen and macrophages in the liver are the main cells taking up adenovirus in intraperitoneally infected mice, and $CD11c^+CD11b^+$ cDCs in the spleen are the major source of a robust IFN-αβ response [16]. In our previous *in vitro* experiments, using blocking antibodies and macrophages generated from knockout mice, and in over-expression studies, we have shown that the macrophage receptor with collagenous structure (MARCO), a member of the class A scavenger receptors, is a major macrophage receptor for Ad with hexon, a major Ad capsid protein, as its binding partner [17, 18]. MARCO is expressed only on a small number of macrophage subsets such as alveolar (AM) [19] and spleen marginal zone macrophages (MZM) [20] but can be induced in macrophages normally not expressing MARCO by some inflammatory stimuli [21]. Notably, some investigators have reported the expression of MARCO on cDC [22–25]. The importance of MARCO for adenovirus uptake in vivo and hypersensitivity induction in adenovirus-infected mice has not yet been studied.

Two signaling pathways emanate from the LPS-activation of TLR4: the MyD88-dependent route required for induction of pro-inflammatory cytokines, such as TNF-α and IL-6, and the TRIF-dependent route, which

is essential for the induction of IFN-αβ and an optimal pro-inflammatory cytokine response, independent of IFN-αβ involvement. In this investigation, we used species C Ads [16] to study the role of macrophages expressing or lacking MARCO in Ad-induced hypersensitization to LPS, specifically in the LPS-induced TNF-α, IL-6, IL-10, and IFN-αβ responses of naïve and Ad-infected mice. The R-LPS of *S. minnesota* R595 was used, which allows the evaluation of the role of CD14 in the production of the unambiguously pro-inflammatory TNF-α [26], the anti-inflammatory IL-10 [27], and IL-6 and IFN-αβ, which, depending on the context, have pro- or anti-inflammatory properties [28–30]. Experiments with *in vitro*-generated Max-Planck-Institute (MPI) cells, AM-like non-transformed macrophage lines [31], bone marrow-derived macrophages (BMM), and freshly isolated AM and peritoneal macrophages (PM) exposed either to Ad or recombinant IFN-β suggest that in Ad-infected mice, not all macrophage populations contribute to the enhanced TNF-α and IL-6 overresponse. We also show that the IFN-αβ-mediated pro-inflammatory response, i.e., enhanced TNF-α and IL-6, but not IL-10 response, is inducible in human blood samples exposed to adenovirus-based SARS-CoV-2 vaccines or human recombinant (hr)IFN-β. The local or systemic production of IFN-αβ in adenoviral vector-vaccinated or virus-infected individuals, together with the ubiquitous occurrence of innate immune stimuli, suggests that the adverse immune effects of infection or vaccination are promoted by the development of hypersensitivity. Therefore, more insights into the underlying mechanisms are of interest and are the basis for this investigation.

Material and Methods

Materials

LPS from *S. minnesota* R595 was extracted from parent bacteria and purified as described [32, 33]. The replication-deficient green fluorescent protein (GFP)-expressing human species C adenovirus (Ad) 5 was grown in 991 cells, and the species C Ad2Ts1 virus was grown in A549 cells at the permissive 32°C temperature as described earlier [16]. These viruses were purified and stored as described [16]. The non-fluorescent Ad2Ts1 was used for the infection of IFN-β reporter C57BL/6 IFN-$\beta^{+/\Delta\beta\text{-luc}}$ mice. Otherwise, the GFP-expressing Ad5 was used for infection of macrophages and mice. The AstraZeneca (AZ) – Oxford University non-replicating chimpanzee Ad-based ChAdOx1 (AZ) and the Johnson & Johnson (J&J) non-replicating species D Ad26-based Ad26.COV2.S (J&J) SARS-CoV-2 vaccines were kindly donated by M. J. Hug, Vaccination Center Freiburg, Germany, and the recombinant mouse (rm)IFN-β by Toray Industries (Tokyo, Japan). hrIFN-β (2.8×10^8 IU/mg) was purchased from R&D Systems (Bio-Techne, Germany), recombinant mouse (rm)IL-10 from ImmunoTools (Friesoythe, Germany), neutralizing monoclonal anti-mouse IL-10 receptor and the control antibody from BioXCell (Biozol, Germany), isoproterenol from Sigma-Aldrich (Merck, Darmstadt, Germany), D-Luciferin Firefly from Biosynth (Staad, Switzerland), and Luciferase Assay System from Promega Corporation (Fitchburg, Madison, WI, US).

Mouse Strains

In general, mice of both sexes, 6–12 weeks of age, were used. C57BL/6 (wt), IRF3$^{-/-}$ [34], IRF7$^{-/-}$ [35], and IFN-αβR$^{-/-}$ mice [36] were bred under specific pathogen-free (SPF) conditions at the Max-Planck Institute Freiburg or purchased from the Jackson Laboratory. C57BL/6 MARCO$^{-/-}$ [21] were bred under SPF condition at the University Hospital Rheinisch-Westfälische Technische Hochschule Aachen. C57BL/6 IRF3$^{-/-}$ mice were generated in the Beutler laboratory using the CRISPR/Cas9 system with the *Irf3* (5′-CCA GTG GTG CCT ACA CCC CG-3′) small base-pairing guide RNA. These mice have one base pair deletion in *Irf3* exon 3 (GCCTACACCC(*C*)GGGGAAGGATA). The C57BL/6 Cd14hdl/hdl containing a functionally null allele of *Cd14* [37] and C57BL/6 IRF3$^{-/-}$ mice were maintained at the Animal Resource Center at UT Southwestern Medical Center; heterozygous IFN-β reporter C57BL/6 IFN-$\beta^{+/\Delta\beta\text{-luc}}$ mice [38, 39], kindly provided by P. Staeheli, were bred at the Institute of Virology, Medical Center-University of Freiburg.

Animal Treatment

The influence of the time interval between Ad infection and LPS challenge on LPS-induced TNF-α production was studied earlier [40]. Maximal enhancement of the TNF-α response was detected 16 h after infection. The IL-6 response was also enhanced at this time point. Strongly enhanced level of both cytokines was detectable in plasma 2 h after challenge. Based on this earlier study, we injected mice (3/group, if not otherwise stated) with 10^{10} Ad particles or 0.2 mL PBS/20 g b.w., i.p., and 16 h later with 20 μg LPS/0.1 mL A.dist./20 g b.w., i.p. If not otherwise stated, 2 h after LPS challenge, mice were killed with isoflurane, and heparinized blood and spleens were collected for further investigation. Each experiment was repeated at least once. All animal experiments in this study were in accordance with institutional, state, and federal guidelines, reviewed and approved by the Regieriumspräsidium Freiburg/Br., BW, Germany, Referat 35 (decision number 35/9185.81; SG-08/68) and by the Institutional Animal Care and Use Committee (IACUC) at the University of Texas Southwestern Medical Center Dallas, TX, USA (decision number: 2017102166).

Immunohistochemistry

Mice (3/group) were sacrificed and perfused as previously described [41]). Spleens were postfixed in 3% paraformaldehyde for 6 more hours. Fixed spleens were dehydrated with 15% sucrose in PBS followed by 30% sucrose in PBS for 16 h each, then embedded in optimal cutting tissue cover (OCT), frozen on a liquid-nitrogen-cooled aluminum block, and stored at −80°C. Frozen spleens were sectioned into 8 μm-thick sections using a Leica cryostat, fixed with acetone at 0°C for 5 min, and rehydrated with PBS for 30 min. To block endogenous peroxidase, sections were treated with Peroxo-Block (Invitrogen) for 1 min, permeabilized in PBS containing 5% normal donkey serum and 0.1%

Triton X-100 for 20 min, and then incubated with rabbit anti-luciferase antibody (Fitzgerald, 70C-CR2020RAP) in PBS containing 3% normal donkey serum at 4°C overnight. For detection of luciferase, signal amplification with the TSA fluorescein system (Perkin-Elmer) was performed according to the manufacturer's instructions using a biotin-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch). Anti-MARCO (Clone ED31, BM Biochemicals) or anti-SIGN-R1 (Clone ER-TR9, AbD Serotec) and DyLight488-, DyLight549-, Cy3-, or Cy2-conjugated secondary antibodies (Jackson ImmunoResearch) were used as recommended by the manufacturers. Slides were mounted in DAPI (4′,6′-diamidino-2-phenylindole)-containing IS mounting medium (Dianova). Digital images were taken with an ApoTome fluorescence microscope (Zeiss) using AxioVision software.

Cell Culture

Mouse cells: BMM were generated from bone marrow precursor cells, as described earlier [42]. MPI cells were generated from fetal liver cells in the presence of GM-CSF, as described [31]. AM from naïve mice were isolated by bronchoalveolar lavage with 5 times 1 mL PBS [31, 43]. Peritoneal cells from naïve mice were obtained by lavage with 5 mL PBS and PM enriched by adherence for 2 h. Spleen cell suspensions were obtained by mechanical disintegration. If not otherwise stated, for the induction of cytokines, 5×10^5 MPI cells or PM/mL, 2×10^5 AM/mL, and 10^6 splenocytes/mL were resuspended in RPMI 1640 containing 1% FCS, while 5×10^5 BMM/mL were resuspended in DMEM containing 1% FCS. BMM were cultured at 37°C in a 7.5% CO_2-containing atmosphere and all other cells in a 5% CO_2-containing humidified atmosphere. To measure cytokine secretion, cells were stimulated in flat-bottom 96-well microtiter plates (Thermo Fisher Scientific; 0.2 mL/well) and mRNA expression in 6-well plates (Nunc; 3 mL/well). If not otherwise stated, 10^8 particles Ad, 10^5 IU rmIFN-β, 100 ng LPS, and 1.0 μM isoproterenol in 50 μL solvent/mL were used for the treatment of cells. If not otherwise stated, the levels of TNF-α and IFN-αβ secreted by mouse macrophages were measured after 6 h, and IL-6 and IL-10 were measured 16 h after stimulation. To inhibit IL-10 effects in LPS-stimulated cultures, the anti-IL-10R (1B1.3A) and the recommended control antibody (HRPN) from BioXCell were used.

Human whole blood (WB) cultures: freshly collected heparinized blood samples from healthy donors were diluted 1:4 with RPMI 1640 and stimulated in 12-well plates to measure cytokine secretion or in polystyrene round-bottom tubes (Corning Science, Mexico) if FACS analysis was performed. If not otherwise stated, a 1:5,000 dilution of AZ (1.25×10^5 IfnU/mL) and J&J (4×10^5 IfnU/mL) SARS-CoV-2 vaccines or recombinant human (hr)IFN-β (5×10^3 IU, i.e., 17.857 ng/mL) were used for pretreatment and 100 ng LPS/mL for the stimulation of human blood leukocytes. Human peripheral blood mononuclear cells (PBMCs) were isolated from donors by density gradient centrifugation using Ficoll-Paque PLUS (Sigma). PBMC-derived CD14+ monocytes were selectively isolated utilizing anti-human CD14 MicroBeads (Miltenyi Biotec) according to the manufacturer's recommended protocol.

Cytokine Determination

The cytokines in cell-free culture supernatants were quantified by the following commercially available ELISAs: mouse TNF-α and IL-6 (Uncoated ELISA Kits) and IL-10 Ready Set Go!, second generation (Invitrogen, Thermo Fisher Scientific), IFN-α (PBL Assay Science) and IFN-β (BioLegend Inc.), and human TNF-α, IL-6, IL-10, and IFN-β (DuoSet, R&D). Bioactive mouse IFN-αβ using a reporter L-929 cell line [37] and bioactive mouse TNF-α was determined in a cytotoxicity test using a TNF-α sensitive L-929 cell line in the presence of actinomycin D [44]. To measure the kinetics of luciferase reporter activity in living IFN-$\beta^{+/\Delta\beta\text{-luc}}$ MPI cells, 75 μg/mL D-firefly luciferin potassium salt (Biosynth) was added to the cells, and bioluminescence was measured in the luminometer (Tecan Infinity M200 Pro).

FACS Analysis

Mouse cells: nonspecific binding was blocked by pre-incubation of the cells with anti-CD16/CD32 antibody (clone 93, BioLegend) in PBS supplemented with 1% goat serum. MARCO was detected with purified anti-MARCO antibody (ED31, AbD Serotec) in combination with Alexa Fluor 647-conjugated goat anti-rat IgG (Molecular *Probes). Furthermore, anti-CD11b.PE (M1/70) and anti-CD11c.PE/Cy7 (N418) from Biolegend, anti-SIGN-R1.APC (ER-TR9, AbD Serotec), anti-F4/80.FITC (BM8, Biolegend San Diego, CA, USA), anti-IRF7.PE (MNGPKL, eBioscience,Carlsbad, CA, USA), and anti-IL-10R.PE (1B1.3a; Biolegend, San Diego, CA, USA) were used. The labeled isotype control antibodies recommended by the respective firms were used.

RNA Isolation and Quantitative Real-Time PCR

Total RNA was isolated from cells and spleen using TRIzol (Invitrogen, Carlsbad, CA, USA) employing Direct-zol™ RNA MiniPrep Kit. To avoid DNA contamination, the samples were DNAse treated during RNA isolation according to the Direct-zol protocols. RNA was reverse transcribed into cDNA using iScript (Bio-Rad, Hercules, CA, USA) and random hexamer primers. PCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad). Primers were generated using AmplifX 1.7.0 (https://inp.univ-amu.fr/en/amplifx-manage-test-and-design-your-primers-for-pcr). The reactions were conducted using iCycler (Bio-Rad) and analyzed employing iCycler iQ software (Bio-Rad). Relative gene expression (rE) was calculated by the formula $rE = 2^{Ct}(gapdh)^{-Ct}(target) \times 10.000$. This formula delivers a unit-free factor. Mouse primers: IRF3: f-AAC TGC CAA GCC CCA ATG TGAA, r-TCA CCT CGA ACT CCC ATT GTT CCT, IRF7: f-ACC TCT TGC TTC AGG TTC TGC AGT, r-AGG GTT CCT CGT AAA CAC GGT CCT, IRF3: f-CCA GGT CTT CCA GCA GAC ACT, r-TAG GCT GGC TGT TGG. AGA TGT and IRF7: f-CCA GTT GAT CCG CAT AAG GT, r-AGC ATT GCT GAG GCT CAC TT, IL-6: f-AGA CAA AGC CAG AGT CCT TCA GAG, r-5GAG AGC ATT GGA AAT TGG GGT AGG, TNF-α: f-AGG GAT GAG AAG TTC CCA AAT GGC, r-GGC TAC AGG CTT GTC ACT CGA AT, IFN-β: f-CCT TTG CAC CCT CCA GTA ATA G, r: GAC GGA GAA GAT GCA GAA GAG T GAPDH: f-CAC CAG GGC TGC TTT TAA CT, r-GAT CTC GCT CCT GGA AGA TG. Human primers: IRF3: f-ACC AGC CGT GGA CCA AGA G, r-TAC CAA GGC CCT GAG GCA C, IRF7: f-TGG TCC TGG TGA AGC TGG AA, r-GAT GTC GTC ATA GAG GCT GTT GG, GAPDH: f-CATGAGAAGTATGACAACAGCCT, r-AGTCCTTCCACGATACCAAAGT.

Data Analysis and Statistics

Data in figures are presented as mean; error bars show SD. Statistical analysis was performed with the unpaired *t* test using Graph Pad Prism 9.2.0 version software (*p <0.05, **p < 0.01, ***p < 0.005).

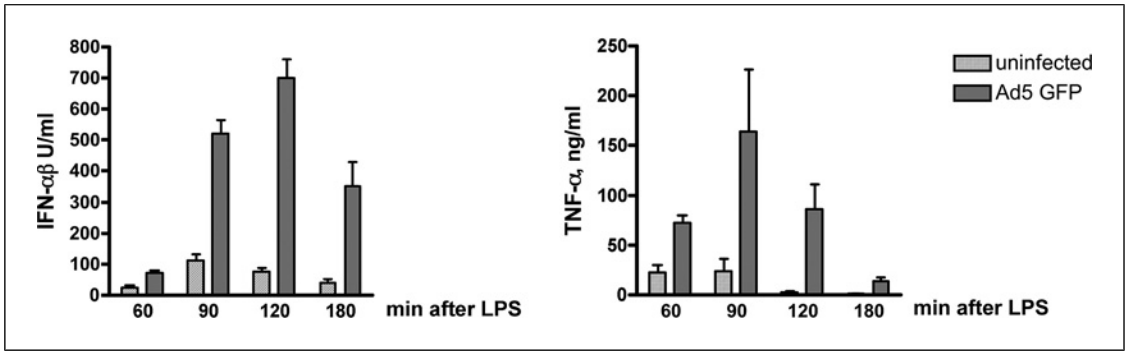


Fig. 1. Enhancement of IFN-αβ and TNF-α response to LPS in adenovirus-infected mice. Groups of three C57BL/6 mice were injected with Ad or PBS and challenged 16 h later with LPS or H_2O. Heparinized blood samples for cytokine measurement were collected at the indicated times after LPS treatment. IFN-αβ and TNF-α were determined by specific bioassays. A representative experiment of 2 is shown.

Results

LPS Hypersensitivity Induced by a Human Adenoviral GFP-Expression Vector (Ad) Includes the Overproduction of Type I IFNs

The effect of Ad pre-infection can be dramatic, moderate, or minimal on the LPS-triggered levels of different cytokines [16, 40]. The production of IFN-αβ has not been studied yet under these conditions. Figure 1 shows that mice infected with Ad and challenged with LPS 16 h later overproduced not only as expected TNF-α but, in addition, dramatically more IFN-αβ. This is an important finding since IFN-αβ, like TNF-α, is also a mediator of LPS toxicity [45].

MARCO Expressed on Macrophages and cDC in the Spleen Is Important for the Uptake of Ad

In vitro experiments with MPI cells and BMM identified the scavenger receptor MARCO as being crucial for adenovirus entry in macrophages and subsequent IFN-αβ production [17]. The spleen is a major site for virus uptake by macrophages and cDC in adenovirus-infected mice [16, 20]. To assess the importance of MARCO for Ad uptake in primary cells, we obtained splenocytes by mechanical disintegration of spleens from wild-type (wt) and MARCO$^{-/-}$ mice. These cells were infected with Ad, and the GFP expression in cells of myeloid origin (CD11b$^+$ cells) was analyzed by flow cytometry. As shown in Figure 2a and online supplementary Figure S1 (for all online suppl. material, see https://doi.org/10.1159/000538282), the relative composition of the myeloid cell population in the spleen from wt and MARCO$^{-/-}$ mice was very similar. Both contained 12–13% CD11b$^+$/SIGN-R1$^+$/CD11c$^+$ (marginal zone macrophages; MZM), 5% CD11b$^+$/SIGN$^-$R1$^-$/CD11c^{++} (cDC), and 60% CD11b$^+$/SIGN-R1$^-$/CD11c$^-$ cells containing other macrophages present mainly in the splenic red pulp (RPM). Analysis of GFP expression in infected wt cells showed that more than 20% of MZM and cDC, but only 5% of the RPM, were GFP$^+$. In infected MARCO$^{-/-}$ cells, the number of GFP-expressing MZM was reduced by 75%, in cDC by 66%, and in RPM by 40% (shown in Fig. 2b). The results demonstrate the importance of MARCO for the uptake of Ad by MZMs and CD11c+ DC, 2 cell types crucial for Ad clearance in the spleen [20].

MARCO Deficiency Inhibits the Establishment of Ad-Induced LPS Hypersensitivity

We next investigated whether the impaired Ad uptake in MARCO$^{-/-}$ mice hampers the sensitization to LPS. Wt and MARCO$^{-/-}$ mice were infected or mock-infected with Ad and challenged with LPS 16 h later. The LPS-induced TNF-α and IFN-β responses were determined in plasma 2 h after challenge. As shown in Figure 2c, injection of LPS into mock-infected wt and knockout mice induced low, comparable levels of both cytokines in both types of mice, indicating that MARCO deficiency does not affect the normal LPS response. In agreement with earlier studies, we found a strongly enhanced TNF-α response to LPS after infection of wt animals [16, 40], which was much less enhanced after infection of mice lacking MARCO. Furthermore, in the absence of MARCO, the Ad-infected mice did not overproduce IFN-β upon LPS challenge (shown in Fig. 2c). In vitro experiments confirmed that only Ad-exposed wt, but not MARCO$^{-/-}$ alveolar macrophage-like MPI cells, the latter lacking the sensitizing IFN-αβ response to Ad [17], overproduce IFN-αβ following LPS stimulation (shown in Fig. 2d).

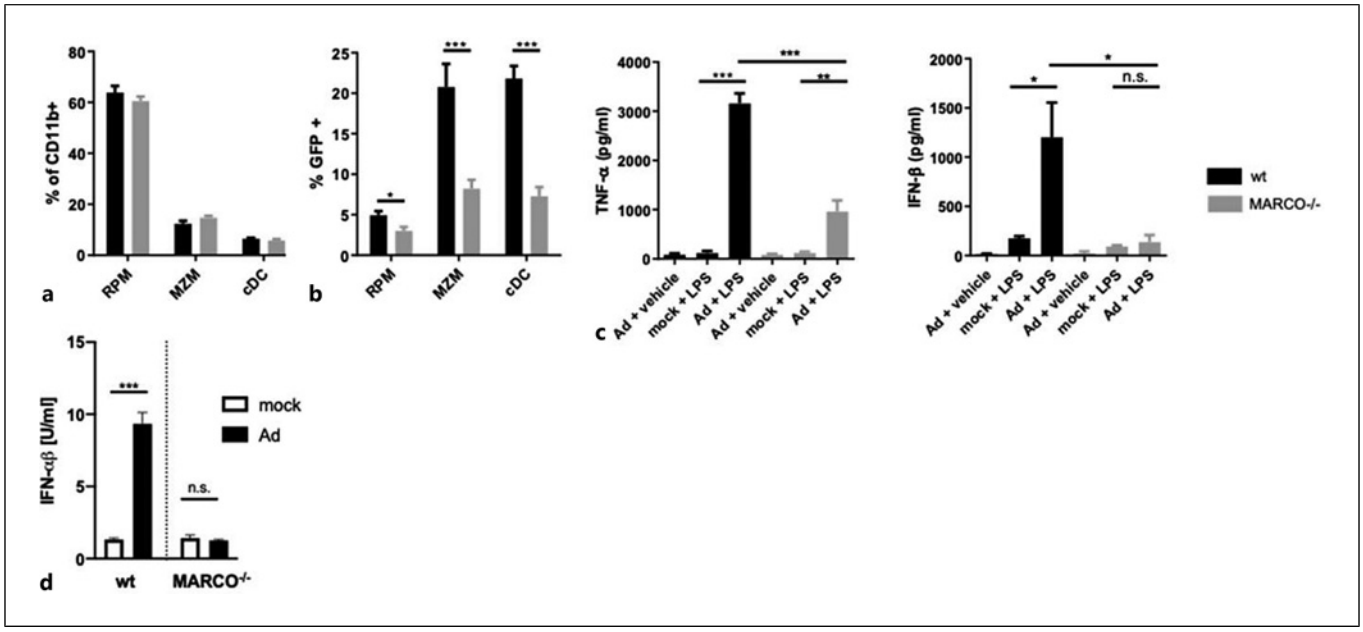


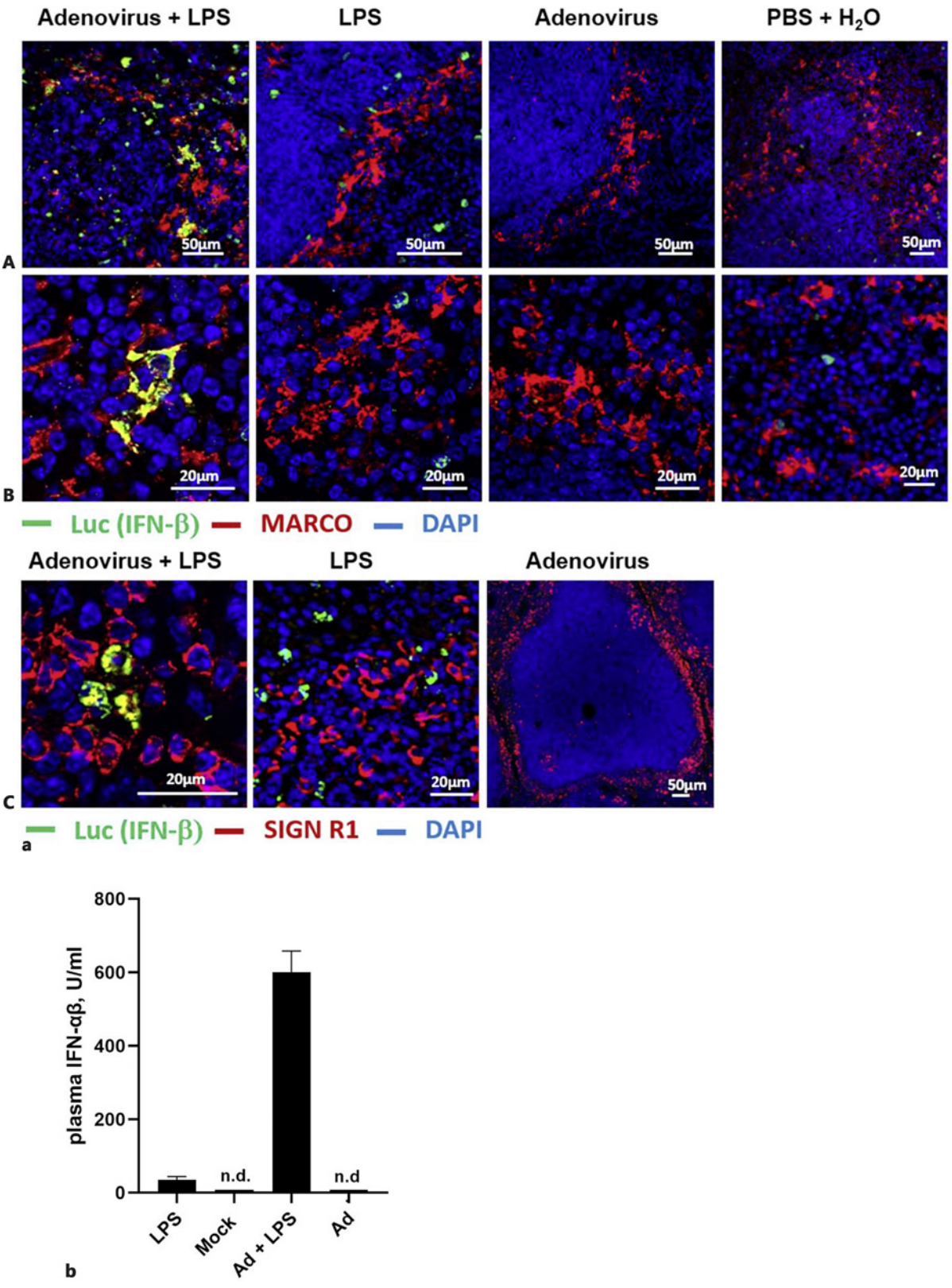
Fig. 2. Requirement of scavenger receptor MARCO for the uptake of Ad by MZM and cDC and for the development of Ad-induced hypersensitivity to LPS. Splenocytes from naïve wt and MARCO$^{-/-}$ mice (7/strain) were obtained by mechanical disruption. **a** Quantification of macrophages and cDC: splenocytes of both strains were stained with anti-CD11b.PE, anti-CD11c.PE/Cy7, and anti-SIGN-R1.APC, and the CD11b^{+} population was further analyzed by flow cytometry with SIGN-R1^{-}/CD11c^{-} (RPM), SIGN-R1^{+}/CD11c^{+} (MZM), and SIGN-R1^{-}/CD11c^{++} (cDC). **b** GFP expression after Ad infection. Splenocytes were infected with the GFP-encoding Ad for 16 h, and the GFP expression in CD11b^{+} cells was analyzed by flow cytometry. **c** TNF-α and IFN-β responses to LPS in vivo. Wt and MARCO$^{-/-}$ mice were injected with Ad or PBS and challenged 16 h later with LPS or H_2O. Two hours after challenge, heparinized blood was collected for cytokine determination by specific ELISA (pooled results from two experiments with 3 mice/group). **d** IFN-αβ response to LPS in Ad-infected MPI cells. Wt and MARCO$^{-/-}$ MPI cells were treated with Ad or remained untreated for 16 h, washed, and stimulated with LPS for an additional 6 h. IFN-αβ was determined with a bioassay. U, relative luminescence units.

Ad Infection Converts Marginal Zone Macrophages to IFN-β-Producing Cells upon LPS Challenge

There is a consensus that IFN-β is the main type I IFN induced in murine macrophages by LPS [46]. To study the effect of Ad on LPS-induced IFN-β production in vivo, we used IFN-β$^{+/\Delta\beta\text{-luc}}$ mice in which one IFN-β allele contained a luciferase ORF inserted in-frame into the IFN-β gene [38]. This allows luciferase expression to be a reporter for IFN-β expression in situ as well as measuring IFN-αβ in plasma. By immunostaining for luciferase, MARCO, and SIGN-R1, we could identify cells producing IFN-β (luciferase positive) in the spleens of mock- and Ad-infected mice after LPS treatment. SIGN-R1 and MARCO are surface antigens characteristic for MZM [47, 48]. No luciferase expression was found in the spleen 16 h after mock or Ad infection (Fig. 3a), and no bioactive IFN-αβ was seen in the plasma (Fig. 3b), respectively. As shown in Figure 3a, b, low levels of IFN-αβ in the plasma and some luciferase-expressing SIGN-R1- and MARCO-lacking cells, mainly in the red pulp, were detectable 2 h after injection of LPS. In contrast, a strong increase in plasma levels of IFN-αβ and the appearance of cells strongly expressing luciferase, SIGN-R1, and MARCO were seen in the marginal zone of Ad-infected mice after challenge with LPS. Thus, MZMs, which played no detectable role in the IFN-β response to LPS in mock-infected mice, became significant IFN-β producers in infected animals. This shows that the Ad-infected MZM became an important source of type I IFN overproduction.

Ability of Ad and IFN-αβ to Enhance the TNF-α and IL-6 Response to LPS Depends on the Macrophage Subset

Macrophages are the major source of cytokines in LPS-injected animals. As shown in Figure 4a, adenovirus infection enhances the TNF-α response to LPS in alveolar macrophage-like MPI cells similarly to the Ad-LPS hypersensitivity shown already earlier in vivo. The absence of enhanced TNF-α response in infected IFN-αβR$^{-/-}$ MPI cells (shown in Fig. 4b) shows that the sensitization in these MARCO-expressing cells is a result of autocrine



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activation by Ad-induced IFN-αβ. Notably, only certain macrophage subtypes express MARCO. Macrophages lacking MARCO, such as BMM (online suppl. Fig. S2, bottom left) or RPM (Fig. 3) show impaired Ad uptake and IFN-αβ response ([17] and Fig. 2b), which excludes an IFN-αβ-mediated autocrine sensitizing effect. However, since in mice the Ad-induced IFN-αβ circulates for several hours [16], also a paracrine sensitization of MARCO-lacking macrophages to LPS in infected mice could be considered. To test this, we pretreated different macrophage types, including MPI cells, BMM, alveolar (AM), and peritoneal macrophages (PM), with rmIFN-β. In a preliminary experiment, we found that priming with 10^5 IU of rmIFN-β exhibits the highest effect on LPS response. This priming dose was then used throughout the study. In MPI cells, we found that the enhancing effect of rmIFN-β on the IL-6 and TNF-α response was most visible 6 h after pretreatment (not shown). If not otherwise stated, this time of pretreatment was maintained for in vitro sensitization throughout the study. The rmIFN-β-pretreated wild-type MPI cells and BMM were stimulated with LPS in parallel, and the cytokine production was determined at 2 h (as mRNA), 6 h, and 16 h (as protein) after LPS addition. Such treatments did not affect the viability of cells. As shown in Figure 5a, b, a significant upregulation of IL-6 and TNF-α mRNA and protein secretion was found in the pretreated MPI cells compared to LPS alone at all time points of investigation, while in BMM, the mRNA expression after 2 h and protein secretion after 6 h remained unchanged, and the secretion after 16 h of stimulation was reduced. Thus, IFN-β appears to enhance the pro-inflammatory responses to LPS in MPI cells but not in BMM. A similar observation was made when we pretreated freshly isolated murine AM and PM with rmIFN-β prior to LPS stimulation. In pretreated AM, both IL-6 and TNF-α responses were enhanced 6 h after LPS, while in pretreated PM, the IL-6 response was reduced and the TNF-α response remained unaffected (shown in Fig. 5c). These results show that not all IFN-αβ-treated macrophage subsets overproduce IL-6 and TNF-α when stimulated with LPS.

Endogenous IL-10 Inhibits Pro-Inflammatory Cytokine Production in LPS-Stimulated BMM, a Mechanism Lacking in Similarly Stimulated MPI Cells

We wondered whether insufficient negative regulation by IL-10 could be responsible for the enhanced LPS-mediated induction of pro-inflammatory cytokines in IFN-αβ-sensitized macrophages. IL-10 is an important negative regulator of the pro-inflammatory cytokine response to LPS [49, 50]. In agreement with our earlier report, LPS-stimulated MPI cells and AM, in contrast to BMM [31], and as shown here also PM, produce no detectable IL-10 (shown in Fig. 6a). To show that the difference in IL-10 inducibility has an effect on the pro-inflammatory cytokine response, we stimulated BMM and MPI cells with LPS in the presence or absence of blocking IL-10R monoclonal antibodies and determined the IL-6 response. As shown in Figure 6b, blocking IL-10 function strongly enhanced the LPS-induced IL-6 production in BMM but had no influence on the production in MPI cells. Thus, the lack of negative regulation by IL-10 is a plausible explanation for the high TNF-α and IL-6 production in LPS-stimulated MPI cells and is expected to contribute to the enhanced pro-inflammatory cytokine production after infection.

The LPS-induced IL-10 macrophage response involves the prior production of IFN-αβ [51–54]. To test whether an insufficient IFN-αβ response to LPS in MPI cells and AM could explain the impaired IL-10 production, we stimulated the different macrophage types in the presence of rmIFN-β. Also under these conditions, the IL-10 response in MPIs and AM remained absent. Furthermore, treatment of BMM with rmIFN-β had no effect on the LPS-induced IL-10 production, while it reduced the production in rmIFN-β-treated PM (shown in Fig. 6a, b).

In a further experiment, we examined whether an IL-10 response can be elicited in MPI cells at all. It was shown previously that in macrophages, a combination of LPS and cAMP results in a synergistic induction of IL-10 [55]. Therefore, we stimulated MPI cells with LPS and the β-adrenergic receptor agonist isoproterenol that elevates cAMP levels and measured the IL-10 levels in culture supernatants. As shown in online supplementary Figure

Fig. 3. The contribution of splenic macrophages to the IFN-β response in LPS-challenged mock- and Ad-infected mice. IFN-β reporter C57BL/6 IFN-$\beta^{+/\Delta\beta\text{-luc}}$ mice were injected with the non-fluorescent Ad2Ts1 or PBS and 16 h later challenged with LPS. Two hours after challenge, heparinized blood samples for IFN-αβ estimation or spleens for immunohistochemistry were collected and stained, as described in Materials and Methods. **a** Luciferase (IFN-β) expression in splenic tissue. To identify IFN-β-producing macrophages in the spleen, luciferase (Luc, green), MARCO (in **A** and **B**, red), and SIGN-R1 (in **C**, red) expression were visualized in paraformaldehyde-fixed, frozen tissue sections. The nuclei were stained with DAPI. **A** Overview at lower magnification. **B**, **C** Detailed images of the marginal zone. Representative examples are shown. **b** Plasma levels of IFN-αβ. The IFN-αβ in plasma was determined in a bioassay. n.d., not detectable.

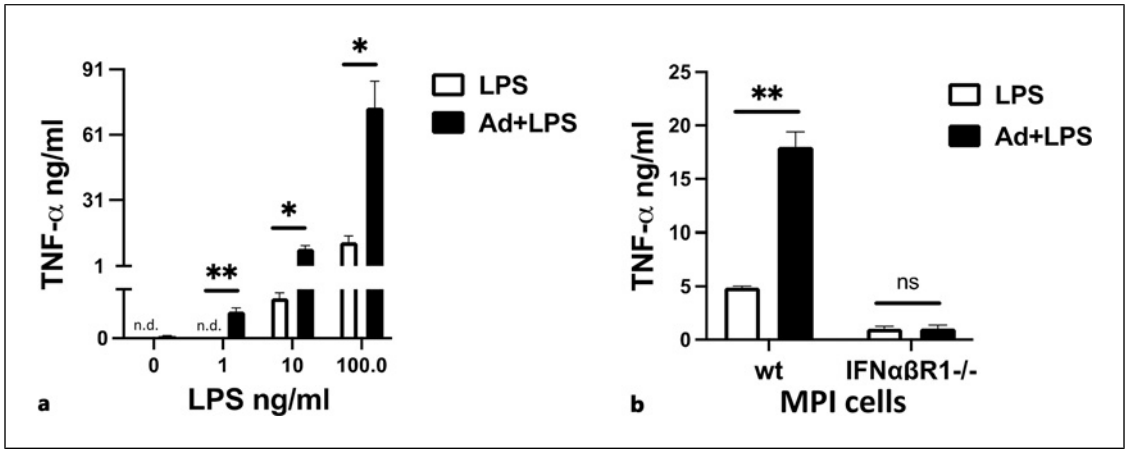

Fig. 4. Ad infection induces IFN-αβ-dependent hypersensitivity to LPS in alveolar macrophage-like mouse MPI cell lines. MPI cells in RPMI containing 10% FCS were infected with Ad and, 16 h later, stimulated with LPS. 6 h later, TNF-α in cell-free supernatants was determined by a bioassay. **a** TNF-α responses and over-responses are dose dependent. Infected and mock-infected wt MPI cells were stimulated with indicated amounts of LPS. **b** TNF-α sensitization requires the presence of the IFN-αβ receptor. Infected and mock-infected Wt and IFN-αβR$^{-/-}$ MPI cell lines were stimulated with 100 ng/mL LPS.

S3, the combination of LPS with isoproterenol resulted in a significant IL-10 response, indicating that this alternative pathway for IL-10 induction is present in MPI cells.

Expression of IL-10R and the Downregulation of the IL-6 Response to LPS by Exogenous IL-10 Is Weaker in MPI Cells than in BMM

Since in mice, LPS-induced IL-10 is also available to cells incapable of producing IL-10 on their own, we investigated the effect of recombinant IL-10 on LPS-induced IL-6 production in MPI cells and BMM. Stimulation with LPS in the presence of rmIL-10 suppressed IL-6 production in both BMM and MPI cells, but less in the latter. The somewhat higher efficiency of suppression in BMM (shown in Fig. 6c) could be due to the higher expression of IL-10R in BMM (shown in Fig. 6d). Our experiments suggest that macrophage subsets with a reduced IL-10 susceptibility could represent an additional source of the enhanced pro-inflammatory cytokine production upon challenge of Ad-infected mice with LPS.

IFN-β Enhances the LPS-Induced IFN-β Response in Both MPI Cells and BMM

As shown above, rmIFN-β exposure enhanced the LPS-stimulated MyD88 signaling-dependent IL-6 and TNF-α production in MPI cells and AM, but not in BMM and PM. We next tested the effect of rmIFN-β on the LPS-stimulated TRIF signaling-dependent production of IFN-β in MPI cells and BMM. We observed that neither control nor rmIFN-β-pretreated MPI cells generated from IFN-β$^{+/\Delta\beta\text{-luc}}$ mice produce luciferase, i.e., IFN-β, without a further stimulation with LPS (shown in Fig. 7a). The time-dependent IFN-β induction in control IFN-β reporter cells peaked 2.5–4.5 h after LPS stimulation. An induction with the same kinetics, but at a higher level, was found after rmIFN-β pretreatment (shown in Fig. 7a), demonstrating the positive effect of type I IFN on the LPS-induced IFN-β response in MPI cells.

To measure the effect of rmIFN-β pretreatment on the endogenous IFN-β response in BMM, we analyzed the IFN-β mRNA expression in pretreated and control BMM 2 h and 6 h post-LPS stimulation (shown in Fig. 7b). In the absence of LPS, the levels of IFN-β mRNA were barely detectable in control and rmIFN-β-pretreated cells. In LPS-stimulated cells, a strong mRNA upregulation was observed after 2 h, and a low but still detectable upregulation was seen after 6 h of stimulation. Pretreatment with rmIFN-β enhanced the LPS-induced upregulation of IFN-β. Together, these results support several earlier studies showing that in macrophages, the initial production of IFN-αβ enhances further production in a positive feedback loop [56–59]. We therefore conclude that the positive feedback loop plays a decisive role also in the upregulation of IFN-αβ response to LPS by endogenously elicited IFN-αβ in Ad-infected MPI cells (shown in Fig. 1d) and MZM of infected mice (shown in Fig. 2).

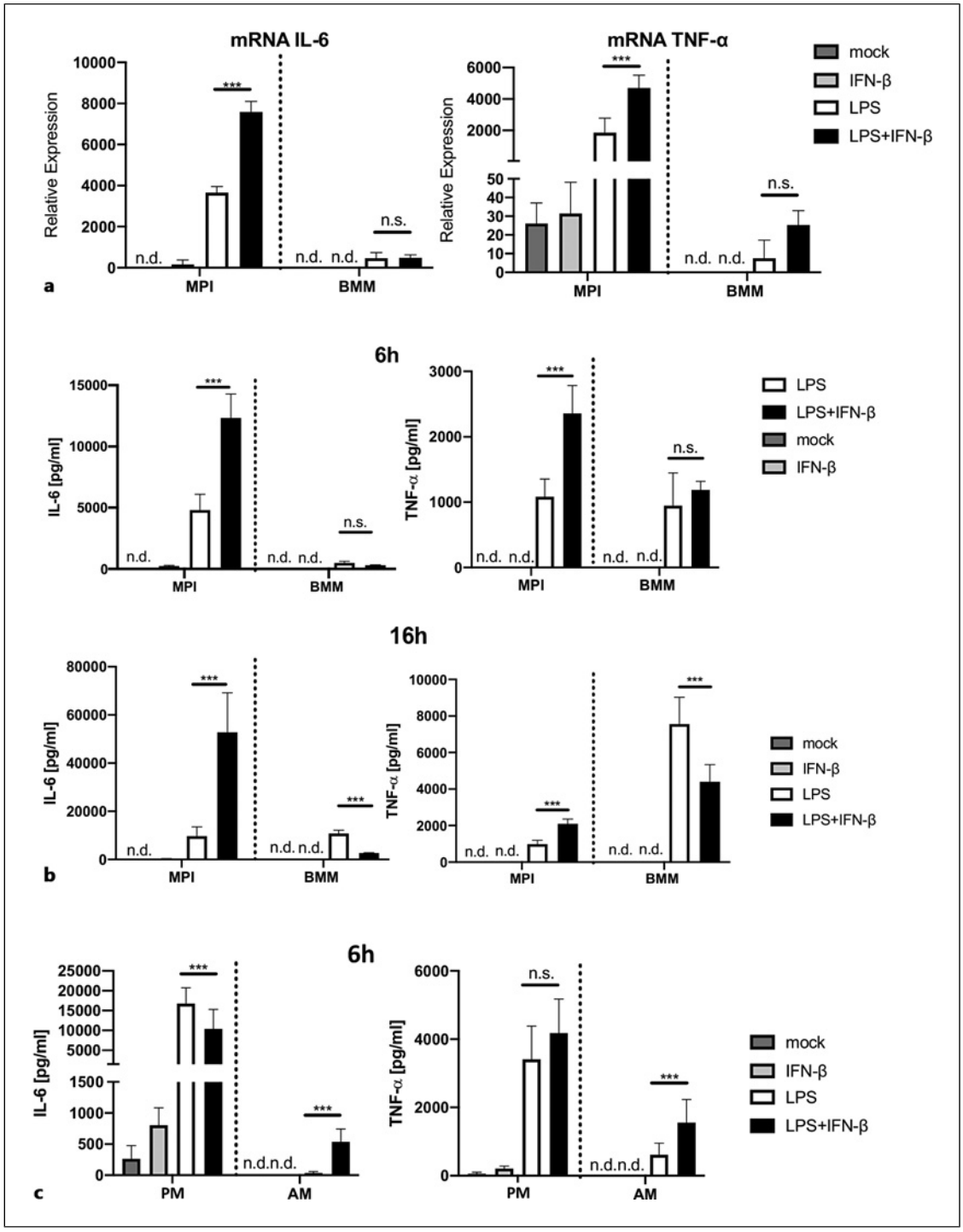

Fig. 5. The ability of IFN-β to enhance LPS-induced TNF-α and IL-6 production is macrophage subset dependent. MPI cells, BMM, PM, and AM were pretreated with rmIFN-β and 6 h later stimulated with LPS. **a** mRNA expression in MPI cells and BMM. Total RNA from MPI and BMM cells was isolated 2 h after stimulation, and the cytokine expression was analyzed by qRT-PCR. Cytokine secretion by MPI cells and BMM (**b**) and PM and AM (**c**). Cytokines were determined in cell-free supernatants 6 h and 16 h after LPS addition in all cells, and MPIs and BMM, respectively, by ELISA. n.d., not detectable. Pooled results of two identical experiments carried out in triplicates are shown.

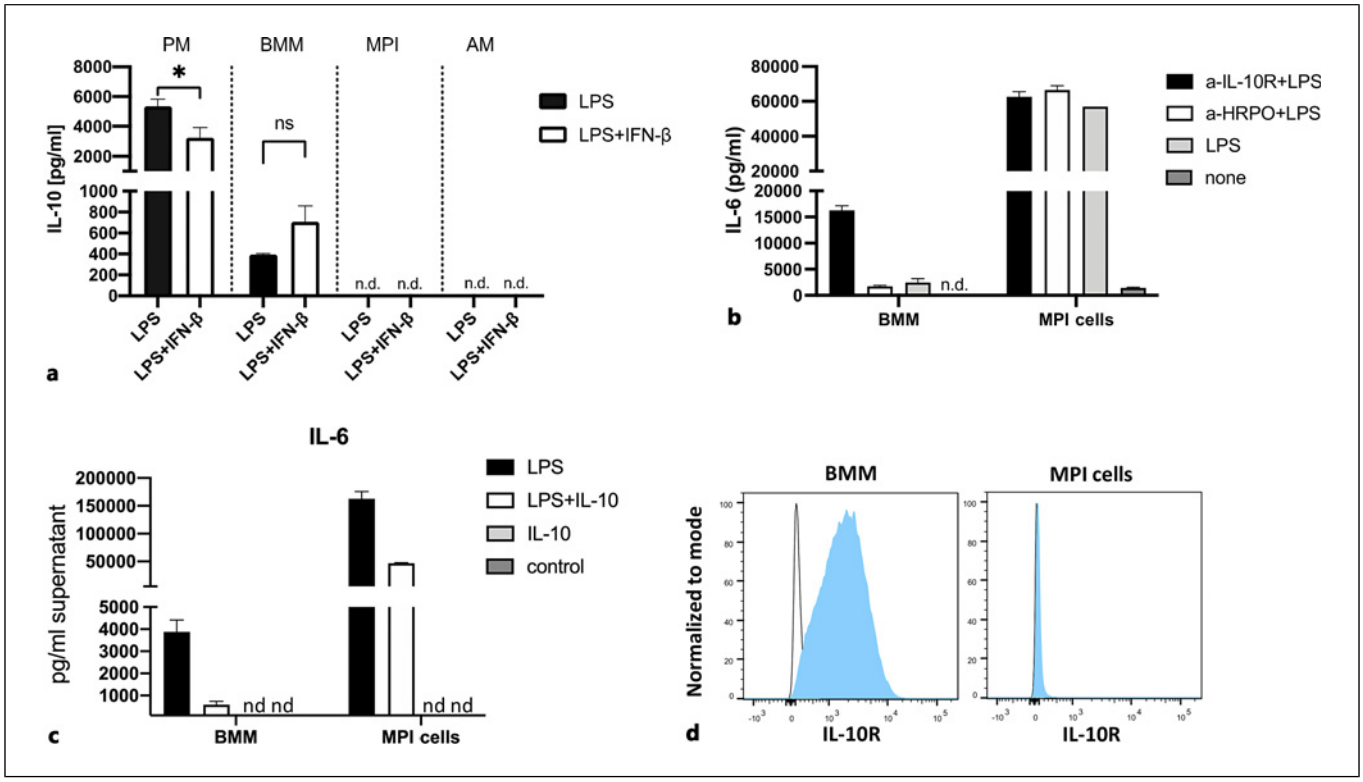

Fig. 6. The ability to produce IL-10 and the height of IL-10R expression regulate the IL-6 response to LPS in mock- and IFN-β-pretreated macrophages. **a** IL-10 response to LPS: MPI's, BMM, PC, and AM were pretreated with rmIFN-β, or remained untreated and 6 h later stimulated with LPS. The IL-10 response in culture supernatants was determined 16 h later by ELISA. IFN-β-stimulated BMM produced, on average, 110 pg IL-10/mL, while all other cell types and unstimulated control cells produced none (not shown in the figure). **b** IL-6 response in the presence of anti-IL-10R. BMM and MPI cells were pretreated with rmIFN-β or remained untreated and, 6 h later, stimulated with LPS in the presence or absence of 1 μg/mL anti-IL-10R or control antibody (anti-HRPO). IL-6 in culture supernatants was determined by a specific ELISA. **c** LPS-induced IL-6 production in the presence of exogenous IL-10. BMM and MPI cells were stimulated with LPS in the presence or absence of rmIL-10 (0.2 μg/mL). The IL-6 in culture supernatants collected 16 h later was determined by ELISA. **d** Cell surface IL-10R expression. The detection of IL-10R on cells was analyzed by FACS using anti-IL-10R.PE (filled blue) or isotype control (open black) antibodies. n.d., not detectable.

IRF3 Is Dispensable for the Ad Enhanced IFN-αβ Response to LPS in MPI Cells

It was shown earlier that the activation of the transcription factor IRF3 is not required for the Ad triggering of IFN-αβ production and LPS hypersensitivity [16], but was needed for the induction of IFN-β by LPS [2, 59]. As shown in Figure 8a and online supplementary Figure S2, unstimulated MPI cells express less IRF3 mRNA than BMM, which is consistent with the weak LPS-stimulated IFN-αβ production of MPI cells relative to that of BMM [31]. Furthermore, IRF3 expression is not upregulated in MPI cells or in BMM 6 h after rmIFN-β pretreatment and therefore cannot explain the enhancement of the IFN-αβ response to LPS in pretreated macrophages. As shown in Figure 8b, the loss of IRF3 reduced further the low IFN-αβ response in mock-treated MPI cells but did not prevent the upregulation of the response in Ad-infected cells. The latter is in agreement with the dispensable role of IRF3 in inducing the sensitization mediator IFN-αβ by Ad [16]. The data show that the LPS-stimulated overproduction of IFN-αβ in Ad-infected MPI cells is largely IRF3 independent.

IFN-β Induces IRF7 mRNA Expression in MPI Cells and Its Upregulation in BMM

IRF7 is another transcription factor known to drive IFN-αβ production. Unlike the ubiquitously present IRF3, IRF7 is either absent or is present in very low quantities in macrophages [60]. As shown in Figure 9 and online supplementary Figure S2, the expression of IRF7 in the control untreated MPI cells was below or

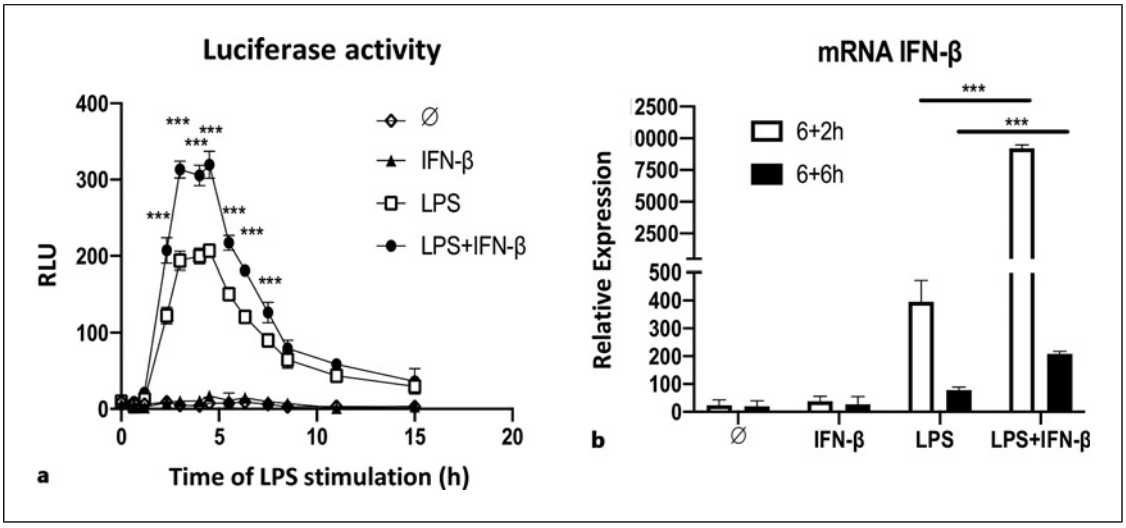

Fig. 7. rIFN-β pretreatment enhances the IFN-β response to LPS in macrophages. **a** The time course of IFN-β production in MPI cells. IFN-$\beta^{+/\Delta\beta\text{-luc}}$ MPI cells were treated with rmIFN-β and 6 h later stimulated with LPS. Cell-associated firefly luciferase activity was measured at the indicated time points after stimulation. RLU = relative luminescence units. **b** Expression of IFN-β mRNA in stimulated BMM. BMM were pretreated with rmIFN-β for 6 h and stimulated with LPS. Total cell RNA was isolated 2 and 6 h after stimulation, and IFN-β mRNA expression was analyzed by qRT-PCR.

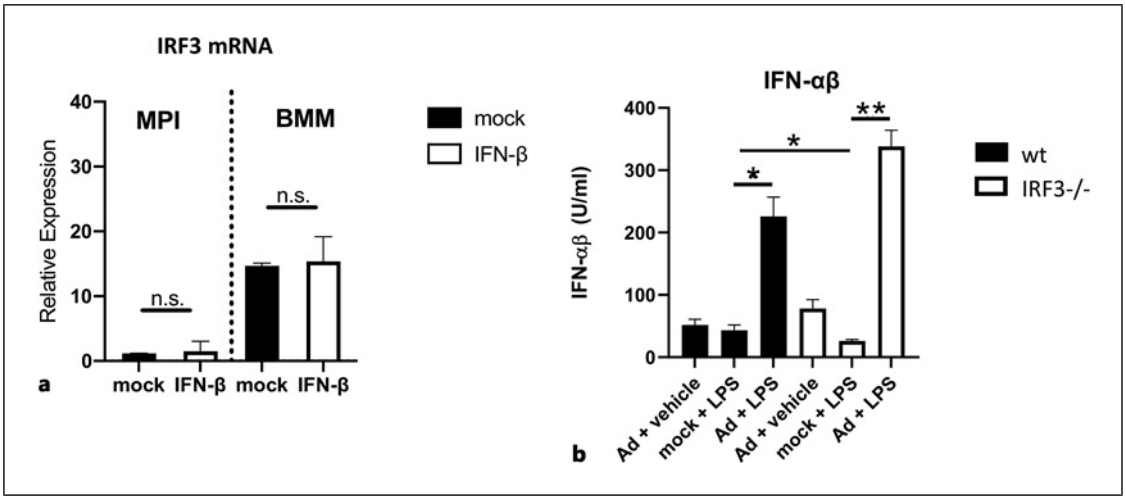

Fig. 8. Role of IRF3 in the induction of IFN-αβ by LPS in rmIFN-β-exposed macrophages. **a** IRF3 mRNA expression in IFN-β-treated MPI cells and BMM. Wt and $IRF3^{-/-}$ MPI cells were cultured with rmIFN-β for 6 h or remained untreated. The total cell RNA was isolated, and the mRNA expression was analyzed by qRT-PCR. **b** IFN-αβ response to LPS in Ad-infected wt an $IRF3^{-/-}$ MPI cells. Wt and $IRF3^{-/-}$ MPI cells were cultured with Ad or mock for 16 h, washed, and stimulated with LPS for a further 6 h. IFN-αβ in cell-free supernatants was determined by a bioassay. Supernatants of mock-treated wt and $IRF3^{-/-}$ cells contained no detectable amounts of IFN-αβ (not shown in the Fig.).

close to the detection limit and low in control BMM. In contrast to IRF3 expression, pretreatment with rmIFN-β was an inducer of IRF7 mRNA expression in both cell types (Fig. 9). Since IFN-αβ-induced IRF7 is needed for maximal interferon expression [16, 61], its upregulated expression most likely drives the IFN-αβ over-response in primed and LPS-stimulated MPI cells and BMM.

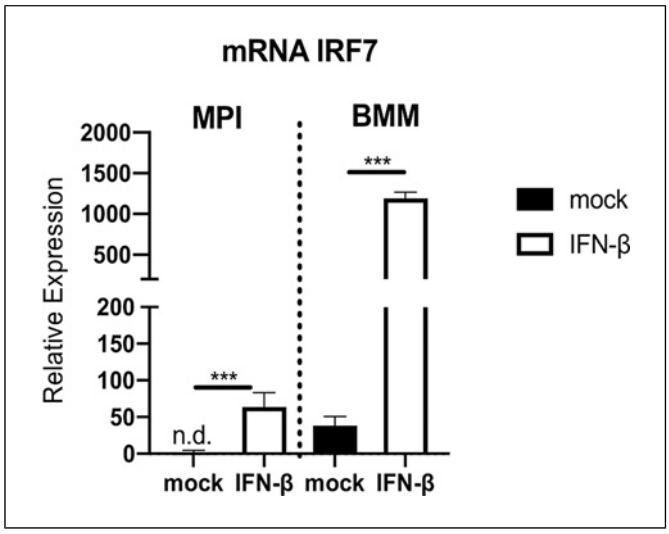

Fig. 9. Treatment of macrophages with rIFN-β upregulates the of IRF7 mRNA expression. MPI cells and BMM were cultured with rmIFN-β for 2 h, and the total cell RNA was isolated. The IRF7 mRNA expression was analyzed by qRT-PCR.

Ad Infection Enables a Very High and Partly IRF3-Independent IFN-α and β Response to LPS in vivo

To assess the in vivo IRF3 requirement for a type I IFN response to LPS, we injected wt and IRF3$^{-/-}$ mice with LPS 16 h after mock or Ad infection and determined the IFN-α and IFN-β production by ELISA. TNF-α production served as a control for sensitization. No production of TNF-α, IFN-α, or IFN-β was seen in both types of mock- and Ad-infected mice without LPS challenge (not shown). The overproduction of TNF-α was induced by LPS in both infected wt and IRF3$^{-/-}$ mice (shown in Fig. 10a), in agreement with our earlier finding that IRF3 deficiency has no impact on Ad-mediated IFN-αβ production [16]. In mock-infected wt and IRF3$^{-/-}$ mice, the IFN-α and IFN-β responses to LPS were at the limit of detectability. The IFN-αβ over-response to LPS in infected IRF3$^{-/-}$ mice was present but significantly lower than that measured in Ad-infected wt mice (Fig. 10a), indicating that the lack of IRF3 was not fully compensated for by other transcription factor(s). Whether cells overproducing IFN-αβ in vivo are all partially dependent on IRF3, or both IRF3-dependent and -independent cells participate in the overproduction of type I IFNs, cannot be decided at present. Figure 10a further shows that Ad infection enhances not only the expected LPS-stimulated IFN-β response but also enables a dramatic IFN-α over-response to LPS in vivo, the latter being less IRF3-dependent than the former. Similarly, a significant but reduced type I IFN overproduction has been also observed when measuring IFN-αβ levels by bioassay in separate experiments with adenovirus-infected LPS-stimulated IRF3$^{-/-}$ mice (Fig. 10c).

Critical Role of IRF7 in the IFN-αβ Overproduction in Ad-Infected Mice, Challenged by LPS

IRF7 is a key transcription factor for type I IFN expression [62] and is critical for the induction of IFN-αβ by Ad [16]. IRF7 mRNA expression was upregulated in the spleens of wt and IRF3$^{-/-}$ mice 18 h after Ad or vehicle administration (Fig. 10b). In Ad-infected mice, this upregulation was not significantly enhanced further by the high IFN-αβ over-response to LPS challenge. Whether a further upregulation of IRF7 mRNA levels by LPS-induced IFN-αβ needs more time or alternatively, the IFN-αβ-induced enhancement of IRF7 mRNA expression in infected mice cannot be further upregulated by a second LPS-induced IFN-αβ stimulus (for example, by reprograming of responsive cells) cannot be decided at present.

To examine whether IFN-αβ exposure enhances IRF7 expression in splenic macrophages, we analyzed the expression of IRF7 in macrophages by flow cytometry after culturing splenocytes with or without rmIFN-β. Extracellular staining of F4/80 followed by intracellular staining with anti-IRF7 was used to assess the expression of IRF7 in splenic macrophages. As shown in online supplementary Figure S4, approximately 19% of these cells expressed IRF7 with an MFI of 1,138. The treatment of the splenocytes with rmIFN-β resulted in both the enhancement of IRF7 staining (MFI = 1,890) and the increase of the number of IRF7-positive macrophages (50%).

We have also tested the LPS-triggered overproduction of IFN-αβ in Ad-sensitized IRF7-deficient mice. In agreement with the importance of IRF7 for the induction of IFN-αβ by Ad, IRF7 deficiency resulted in a heavily impaired IFN-αβ over-response in LPS-treated Ad-infected mice (Fig. 10c).

Differential Need for CD14 Indicates Distinct Cellular Sources of LPS-Stimulated Cytokines in Both Mock- and Ad-Infected Mice

Depending on the LPS chemotype and cell type, the activation of MyD88 signaling may or may not require the help of the LPS-binding protein CD14. The production of cytokines by rough-form LPS (R-LPS) chemotypes *via* the MyD88 pathway is inducible in CD14-deficient BMM and PM but not in CD14-low MPI cells and AM, while TRIF signaling is always dependent on

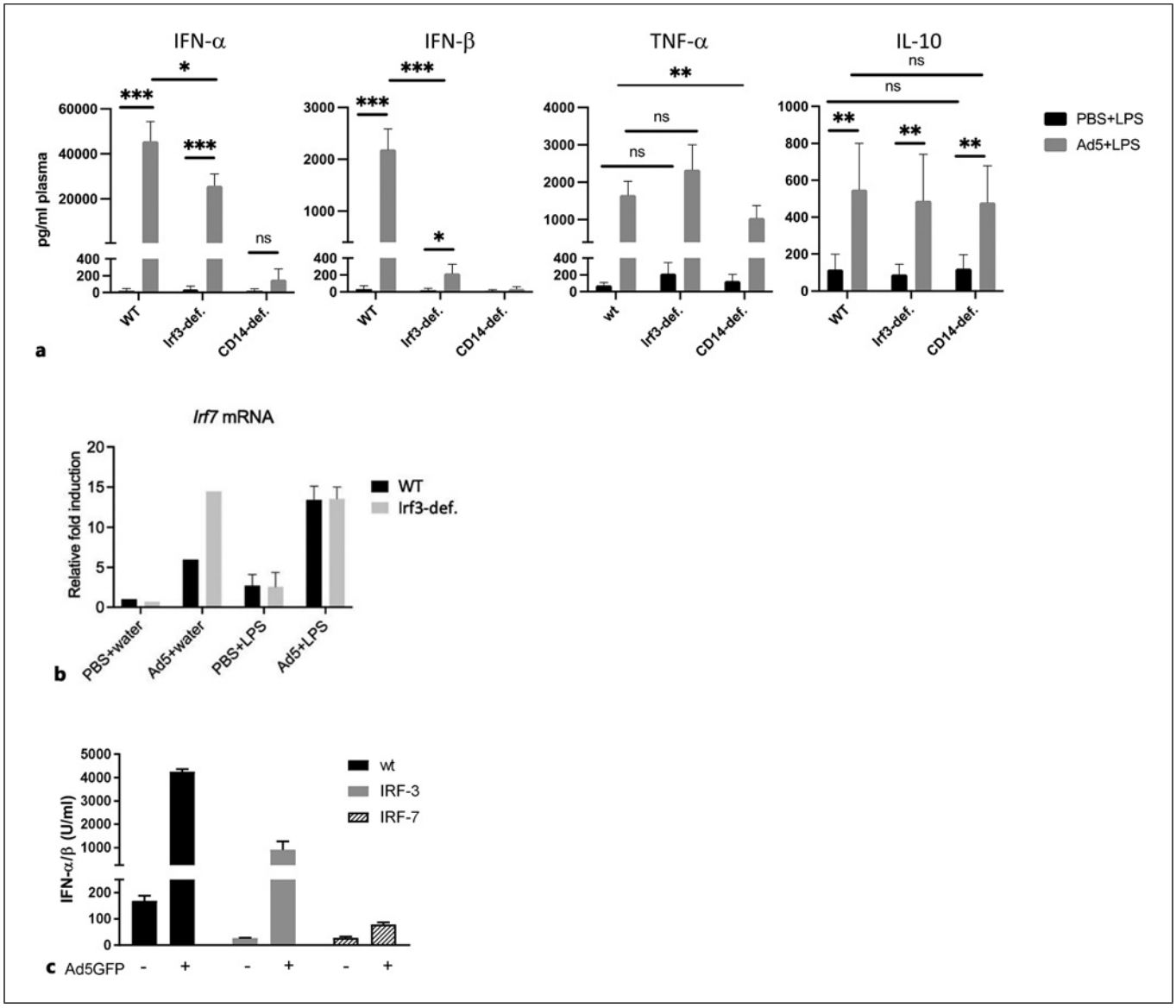

Fig. 10. Effect of IRF3 and CD14 deficiency on the response to LPS in mock- and Ad-infected mice. Wt, IRF3$^{-/-}$, and CD14-deficient mice (5/group) were injected with Ad or PBS and challenged 16 h later with LPS or H_2O. Heparinized blood samples for cytokine determination and spleens for RNA isolation were collected 2 h after the LPS challenge. **a** Cytokine production. Cytokines were determined in plasma by specific ELISA's. **b** IRF7 mRNA expression. The analysis of IRF7 expression by real-time PCR was carried out using total spleen RNA. **c** Wt, IRF3$^{-/-}$, and IRF7$^{-/-}$ were injected with Ad or PBS and challenged 16 h later with LPS. Heparinized blood samples were collected 2 h after LPS challenge, and IFN-αβ in plasma was determined by a bioassay.

CD14 help [31, 37, 63]. Examination of CD14-deficient mice and using R-LPS in this study allowed us to investigate the participation of CD14-dependent (AM-like) and -independent (PM-like) macrophages in the LPS-elicited production of individual cytokines in mock- and Ad-infected mice. As shown in Figure 10a, the TNF-α response to LPS, mediated by the MyD88 pathway, was comparable in mock-infected wt and CD14-deficient mice. This suggests that the sources of TNF-α are CD14-independent and not CD14-dependent cells. The CD14 deficiency, however, significantly lowered TNF-α overproduction in Ad-infected mice, indicating that macrophages requiring CD14 for LPS-induced Myd88 signaling contribute to the enhanced TNF-α response to R-LPS in sensitized animals.

CD14 assists in the translocation of the LPS/TLR4/MD-2 complex from the cell surface into the endosome [64], where the TRIF-IRF3-mediated IFN-β production is induced [65]. As shown in Figure 10a, the IFN-α and IFN-β responses to LPS were absent in both mock- and Ad-infected CD14-deficient mice, which indicates that sensitization by Ad does not abolish the requirement for CD14-assisted TRIF signaling in the induction of IFN-α or IFN-β. IFN-αβ reportedly drives efficient induction of IL-10 by LPS [51, 53, 66, 67]. However, as shown in Figure 10a, comparable IL-10 responses and weak over-responses were triggered by LPS in wt and CD14-deficient mock- and Ad-infected mice, respectively. The results indicate that in both naïve and hypersensitive mice, the IL-10 response is induced by the TRIF- and IFN-αβ-independent pathways and originates from CD14-independent cells.

Adenoviral Vector-Based SARS-CoV-2 Vaccines and Human rIFN-β Differentially Regulate TNF-α and IL-10 Responses to LPS in Human Blood Cells

Adenovirus-based vectors, including the SARS-CoV-2 vaccines from J&J and AZ, are used to immunize humans. To test the influence of these vaccines on the cytokine response to LPS in a human model, we treated heparinized whole blood (WB) samples from healthy human donors with J&J and AZ, for comparison with rhIFN-β, and 24 h later with LPS for a further 24 h period. The determination of IFN-β at the time of LPS addition revealed that all vaccine-treated WB samples contained type I IFN, while the supernatants of mock-pretreated cells were always IFN-β negative (shown in Fig. 11a). The amount of IFN-β found varied depending on the donor and the vaccine used. Figure 11a shows further that although the amount of rhIFN-β used for pretreatment of WB cells was the same for all donors, the remaining amount of IFN-β detectable by ELISA after 24 h of culture varied from donor to donor. The reason for these differences was not addressed in this study. Pretreatment with the vaccines or rhIFN-β upregulated the LPS-stimulated TNF-α and IL-6 production in WB cultures of all donors and, in most cases, downregulated the IL-10 response (shown in Fig. 11b). The efficacy of pretreatment varied and was dependent on the individual donor, the agent used for pretreatment, and the cytokine measured (shown in online suppl. Fig. S5). As IRF3 and IRF7 can influence the production of type I IFNs in adenovirus and LPS, we assessed the basal mRNA expression levels of these genes in PBMCs and CD14+ monocytes. Both IRF3 and IRF7 expression were readily detectable, and the differences in the individual expression levels of IRF7 were significantly higher than those of IRF3 across the five studied individuals (online suppl. Fig. S6).

Discussion

IFN-αβ elicited by viral pathogens or virus-based vectors induces hyperreactivity to innate immune stimuli, including LPS, with potentially harmful consequences [4, 8, 12, 16]. Nevertheless, the underlying cellular mechanisms are largely unclear. In this study, we investigated the effect of Ad-induced or recombinant IFN-αβ on LPS-induced cytokine production in mice, in different *in vitro*-generated or freshly isolated mouse macrophages, and ex vivo in human blood samples. In the mouse model, we used replication-deficient species C adenoviruses, and for human cells, adenovirus-based SARS-CoV-2 vaccines.

Since macrophages and cDC are major immune cell targets for Ad entry and subsequent production of the sensitizing IFN-αβ in intraperitoneally infected mice, we first aimed to identify the receptor involved in Ad uptake and sensitization. Several paths of adenovirus entry into macrophages, such as direct uptake *via* β3 integrin and MARCO [17, 18] and indirect uptake *via* receptors for soluble factors binding to the virus, were described in the past. The latter include natural antibodies, complement proteins C3 and C4, and coagulation factor X [68]. The analysis of ex vivo Ad5-GFP-infected splenocytes from wt and $MARCO^{-/-}$ mice showed that $MARCO^{+/+}$ MZM and cDC but not $MARCO^{-/-}$ MZM and cDC or MARCO-negative RPM were the main GFP-expressing cells. These results confirm the crucial role of MARCO in Ad uptake by macrophages and indicate that this receptor plays a crucial role in Ad entry in MZMs and splenic cDC. Impaired TNF-α overproduction and the absence of an IFN-αβ over-response to LPS in Ad-exposed MARCO-deficient macrophages and mice revealed the importance of MARCO-expressing and IFN-αβ-producing cells for the development of hypersensitivity to LPS. The significantly reduced but still present TNF-α over-response in $MARCO^{-/-}$ mice is most probably induced by IFN-αβ produced by cells in which Ad entry occurs *via* additional Ad receptors. The obvious difference between the reduced TNF-α and the lacking IFN-β over-response in infected MARCO-deficient mice is interesting. Whether it indicates a difference in the threshold levels of IFN-αβ required for the upregulation of the TNF-α (lower) and the IFN-β (higher) response or suggests that IFN-β, unlike the TNF

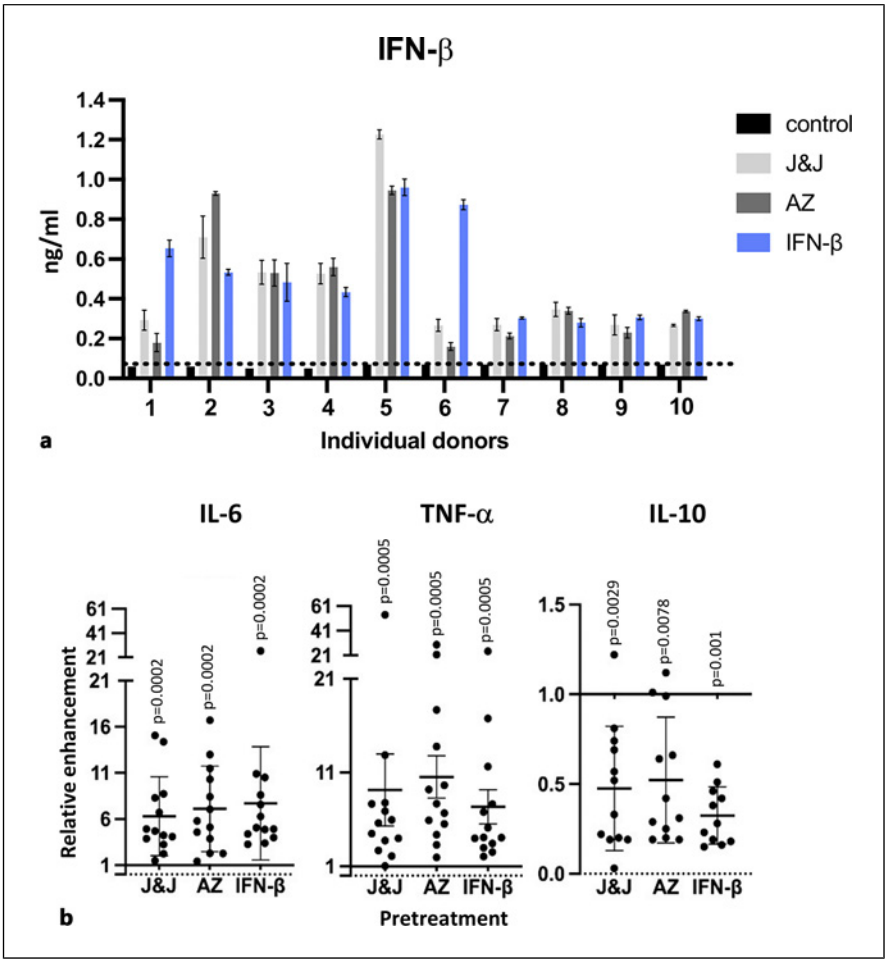

Fig. 11. Adenoviral vector-based SARS-CoV-2 vaccines induce type I IFN and, like exogenous IFN-β, differentially regulate LPS-induced cytokine responses in human whole blood. WB samples from healthy donors were pretreated with J&J, AZ vaccines, or hrIFN-β, or remained untreated and 24 h later stimulated or not with LPS for an additional 24 h, as described in Material and Methods section. **a** IFN-β determination at the time of LPS treatment. IFN-β was determined in the culture supernatants of 10 donors 24 h after the hrIFN-β pretreatment by ELISA. Supernatants of mock-pretreated WB cells contained no detectable IFN-β (control). Dotted line: detection limit. **b** TNF-α, IL-6, and IL-10 responses to LPS after vaccine or hrIFN-β pretreatment. The cytokine amounts were determined in whole blood supernatants from 12–13 individual donors at the end of the experiment (48 h) by ELISA. Values found in agent-pretreated, LPS-stimulated cell supernatants relative to those found in mock-pretreated and stimulated once are shown. No TNF-α, IL-6, and IL-10 was found in completely unstimulated and vaccine- or rIFN-β-only-pretreated whole blood cells (not shown).

over-response, originates exclusively from MARCO$^{+/+}$ cells, has to be addressed in future studies.

Not all macrophages express MARCO. In mice, MARCO expression is restricted to macrophages in the splenic marginal zone, lung alveoli, and to distinct populations of macrophages in lymph nodes [69, 70]. Notably, BMM and RAW 264.7 cells, two types of mouse cells frequently used in macrophage studies, do not express MARCO, and as a consequence, BMM do not produce IFN-αβ after exposure to Ad [17]. MARCO can, however, be induced on non-expressing macrophages by infection, such as on MARCO-negative Kupffer cells in the liver after exposure to the bacillus Calmette-Guerin or during bacterial sepsis [71]. In our study, the continued absence of immunodetectable MARCO in the red pulp of the spleen of infected mice reveals the inability of Ad infection to induce this receptor on RPM.

While the exposure of MARCO-expressing MPI cells and AM to Ad in vitro induces IFN-αβ production and LPS hypersensitivity in an autocrine fashion, the lack of MARCO precludes the same effect in BMM. In Ad-infected mice, however, IFN-αβ remains in the circulation for several hours [16], which was expected to enable a paracrine sensitization of all macrophages expressing the IFN-αβ receptor. Indeed, increased TNF-α production has been observed in organs both infected and not infected by the virus during Ad hypersensitivity [40]. Against the expectation of a universal sensitizing effect by IFN-αβ, an increased LPS-triggered cytokine production was not found in BMM and PM treated with rIFN-β. On the contrary, reduced TNF-α and IL-6 production was observed in pretreated cells late (16 h) after LPS stimulation. IFN-αβ-mediated inhibition of the TLR4-initiated pro-inflammatory cytokine production in BMM was also recently reported [30]. In addition, IFN-αβ had been found to be involved in several LPS-induced BMM activities in earlier studies [72, 73]. Therefore, published data as well as our own investigation rule out the absence of a functional IFN-αβR on BMM.

In tests designed to explain the differences in the effect of IFN-αβ priming on the pro-inflammatory cytokine induction by LPS in different macrophage subsets, we took into consideration that LPS-induced IFN-αβ was shown to play a prominent role in the subsequent induction of IL-10 [51, 53, 66, 67] and that IL-10 is a negative regulator of the pro-inflammatory cytokine response [49, 50]. LPS-stimulated MPI cells, in contrast to BMM, do not secrete significant amounts of IL-10 [31]. By showing a strong enhancement of LPS-induced IL-6 production in anti-IL-10R-treated BMM but not in MPI cells, we provide evidence that IL-10 does not regulate the pro-inflammatory response in MPI cells. Importantly, macrophage subsets distinct in their ability to produce IL-10, as shown here with freshly isolated PM and AM, also exist in mice. The reason for the impaired IL-10 response in MPI cells and AM is not yet known. By showing that exogenous IFN-β alone or in combination with LPS does not induce IL-10 in MPI cells, we eliminated the possibility that insufficient IFN-αβ production is responsible for their impaired IL-10 response. Further doubt about any significant contribution of IFN-αβ to IL-10 induction came from our in vivo experiments in which we compared the IL-10 plasma levels in mock- or Ad-infected wt, IRF3- and CD14-deficient mice challenged with LPS. Despite the fact that IRF3 and CD14 deficiency cause a reduction or complete loss of IFN-αβ production, neither of the two had an impact on the IL-10 response to LPS. It should be noted, however, that the role of IFN-αβ in the IL-10 response of target cells to LPS is unclear. Although IFN-αβ is a recognized intermediate in IL-10 induction, an inverse effect, namely the inhibition of IL-10 production by IFN-αβ in LPS-stimulated mouse RAW 264.7 cells, mouse thioglycolate-elicited PM, and in human monocyte-derived macrophages, was described earlier as well [74]. Such an inhibitory effect was not observed in our murine experiments but was visible in human blood cells pretreated with recombinant IFN-β. It is highly probable that this effect contributes to the enhanced TNF-α and IL-6 response in the pretreated human cells.

Interestingly, while the IL-10 response to LPS alone is impaired in MPI cells, we show that, in synergy with the cAMP-inducing agent isoproterenol, IL-10 is inducible in these cells (shown in online suppl. Fig. S2). Synergies between LPS and different co-stimuli, including cAMP, IgG immune complexes, or apoptotic cells, were shown to lead to IL-10 induction in several earlier studies [55, 75, 76]. In vivo, in mock- or Ad-infected mice injected with LPS, the presence of such co-stimuli may also support the IL-10 response in macrophages, which do not primarily produce IL-10, and thus contribute to the downregulation of the LPS-induced pro-inflammatory response. Also, differences in the expression of the cell-associated IL-10 receptor may play a role in the regulatory effect of IL-10. The reduced ability of hrIL-10 to suppress the IL-6 response in LPS-stimulated MPI cells compared to similarly stimulated BMM may result from lower IL-10R expression in the former. The identification of macrophages differing in their ability to produce IL-10 and express IL-10R, and the assessment of their contribution to the pro-inflammatory response to LPS, should be the subject of further research.

Unlike the TNF-α and IL-6 responses, the IFN-αβ response to LPS increased after IFN-αβ priming in both MPI cells and BMM. IFN-β induction was also found in MZM of Ad-infected mice challenged with LPS. These findings are in accordance with the known positive feedback of IFN-αβ on its own production [16, 58, 61]. Interestingly, the enhancement was especially strong in LPS-stimulated macrophages, which, in the absence of sensitization, produced very low (MPI cells, AM) or undetectable (MZM) amounts of IFN-αβ. The underlying reason for these findings remains elusive at this time.

Interestingly, Ad infection in mice enabled not only a high IFN-β response but, in addition, a very strong IFN-α overproduction. In mice, the IFN-α family includes 14 members with approximately 80% sequence similarity. The ELISA used to detect IFN-α did not distinguish between individual interferon subtypes. Although all type I IFN bind and activate a single IFN-αβ receptor, variation in their receptor binding activity is thought to be responsible for many differences in their biological activity. In general, due to its highest binding activity, IFN-β is the most active member of type I IFN [9]. The contribution of each individual family member to the damaging effects of LPS in hypersensitive mice and humans is of interest and a potential subject of follow-up investigations.

It is generally accepted that the constitutively expressed transcription factor IRF3, activated *via* the TRIF signaling pathway, is needed for the induction of IFN-β by LPS [59, 77, 78]. The acquired ability to overproduce IFN-αβ in IRF3$^{-/-}$ infected MPI cells, and especially of IFN-α in infected IRF3$^{-/-}$ mice, shows that additional Ad-induced transcription factor(s) decisively contribute to the strongly enhanced IFN-αβ response. The upregulation of IRF7 expression was shown to be especially involved in the production of the IFN-α members of the IFN-αβ family by positive feedback [16, 61]. Thus, a likely candidate for this regulation is IRF7, a transcription factor closely related to IRF3, which plays a crucial part in the feedforward loop driving the maximal IFN-αβ response [16, 61, 62, 79, 80]. The expression of

IRF7 in most cells is generally very low [35, 81], but becomes upregulated during viral infection [61]. This is supported by the fact that in our study, IFN-αβ priming upregulated IRF7 expression in vitro in MPI cells, BMM and splenic macrophages, and in the spleen of Ad-infected mice. Loss of the feedforward mechanism involved in IFN-αβ production in Ad-infected IRF7$^{-/-}$ mice which mainly affects the IFN-α response [16], resulted in an impaired TNF-α over-response to LPS. More recently, it was shown that, in addition to IRF3, IRF7 also participates in the LPS-mediated IFN-αβ response in macrophages, in vitro, and in mice in vivo [82]. It is therefore highly probable that the enhancement of IRF7 expression in macrophages by Ad infection contributes to the enhanced production of IFN-αβ elicited by subsequent LPS stimulation. This mechanism also explains the absence of LPS-induced IFN-αβ over-response in Ad-infected IRF7$^{-/-}$ mice and the powerful IFN-α response to LPS in Ad-infected mice, which was only moderately lowered by the disruption of the IRF3 gene. The crucial role of IRF7 in IFN-α induction was reported earlier [61].

The need for CD14 assistance in the activation of MyD88 signaling depends on the LPS chemotype (S or R) and macrophage subset [31, 37, 63]. Comparing the activity of R-LPS in wt and CD14-deficient mice allowed us to evaluate the contribution of CD14-dependent and -independent macrophages to the induction of distinct cytokines before and after Ad sensitization. LPS-injected mock-infected wt and CD14-deficient mice exhibited comparable TNF-α responses, indicating that CD14-independent cells alone drive normal levels of TNF-α production in vivo. In an earlier study, we found enhanced expression of CD14 on F4/80-positive splenic macrophages in adenovirus-infected mice [16]. Others found a positive effect of IFN-αβ on the expression of CD14 in macrophages [83]. The reduction of LPS-induced TNF-α levels in Ad-infected CD14-deficient mice indicates the contribution of CD14-dependent mechanisms and/or cells to the upregulation of TNF-α overproduction.

Whereas Myd88 activation leading to TNF-α induction takes place on the cell surface, type I IFN induction has been shown to require the CD14-assisted translocation of LPS-associated TLR4 into the endosome and subsequent activation of TRIF signaling [64, 65]. The absence of an LPS-induced IFN-αβ response in both uninfected and infected CD14-deficient mice suggests that the CD14-assisted activation of TRIF signaling is equally necessary for the induction of IFN-αβ after Ad sensitization.

As mentioned above, neither the normal nor the enhanced IL-10 response to LPS by Ad was hampered by a CD14 deficiency in vivo. This enhancement, however, was rather moderate compared to that of TNF-α, IL-6, and IFN-αβ in Ad-infected mice. The disproportionately greater overproduction of the toxic cytokines TNF-α, IL-6, and IFN-αβ relative to IL-10 likely contributes to the adverse effects of LPS in Ad-infected mice [40].

By showing that the regulation of the TLR4-induced cytokine response by Ad-induced IFN-αβ is specific to each cytokine type, enhancing in some cases but inhibitory in others, and varying between macrophage subpopulations, we provide a further example for the functional diversity of macrophage subsets [84, 85]. Housing of animal donors and culture conditions may influence the functions of macrophages. However, the differences in the reactivity to LPS between the rIFN-β-primed BMM and MPI cells were observed independent of the various donors of embryonal precursors for MPI cells and the number of MPI cell passages. The differences in origin (embryonal precursors for MPI cells vs. bone marrow-derived precursor for BMM) and growth factors needed for the generation of MPI cells (GM-CSF) and BMM (M-CSF) are factors potentially involved. Furthermore, the tissue-specific environment is expected to shape macrophage functionality in the steady state and after infection or tissue damage. Importantly, regarding PM and AM, macrophages phenotypically similar to the in vitro-generated BMM and MPI cells were found in vivo. In summary, our study strongly suggests that some, but not all, macrophages contribute to the pro-inflammatory cytokine overproduction in Ad infection/IFN-αβ-sensitized macrophages to innate immune stimuli. Further elucidation of the underlying mechanisms is needed.

Like the TLR4 ligand LPS, other TLR activators, including microbial constituents [46, 86, 87], damage-induced endogenous products [88–90], and exogenous non-microbial substances [91, 92], could be expected to have similar effects in animals and humans infected with adenoviruses or treated with Ad-based vaccines. This may lead to unwanted adverse effects, both at the local and systemic level. Here we show that treatment of heparinized human whole blood with the COVID-19 vaccines from J&J and AZ induces IFN-β, and like treatment with rIFN-β, it enhances the pro-inflammatory TNF-α and IL-6 responses, and mainly inhibits the anti-inflammatory IL-10 response to LPS in blood cells. In an earlier study, an upregulated TNF-α response to LPS was also observed in human peripheral blood mononuclear cell infected with UV-inactivated adenovirus R700 [40]. Although not directly shown, it is very likely that vaccine-induced IFN-αβ is involved in these effects. This is an important result since determination of the cytokine responses to innate

immune stimulation in WB in the presence or absence of IFN-αβ-inducing pretreatment could be used to predict the damaging hyperinflammatory responses occasionally associated with viral infection and vaccination [93]. The level of the LPS-induced cytokine response and the changes caused by the vaccines varied, depending on the donor. The differences in donor reactivity to viral exposure might rely on differences in the basal expression of genes able to influence Ad and LPS-induced cytokine production. Here, we observed variable levels of IRF7 expression in human PBMCs and CD14+ monocytes. We have shown a critical role of IRF7 in IFN production induced by Ad infection, alone or in combination with LPS treatment [16] and this study, respectively. Thus, the observed variations in the basal IRF7 levels of healthy individuals may contribute to the variable responses observed upon vaccine and LPS treatment. However, other factors, genetically determined or dictated by changes in the environment, may also play significant roles. The elucidation of such differences is expected to help in the application of infection-specific therapies and in the prevention of vaccination-elicited adverse effects.

Acknowledgments

We thank J. Ippisch, K. Fehrenbach, A.C. Rühl, and U. Voith for technical assistance and T. Zor for the re-import of the mouse IFN-αβ reporter L-929 cell line. We are thankful to C. Galanos, P. Staeheli, and J. Müller-Quernheim for discussions and continuous support. We are grateful to E. Morresco for critical reading.

Statement of Ethics

All animal experiments in this study were reviewed and approved by the Regieringspräsidium Referat 5 (Regional Board) Freiburg (decision number: 35/9185.81; SG-08/68) and by the Institutional Animal Care and Use Committee (IACUC) at the University of Texas Southwestern Medical Center Dallas (decision number: 2017102166). The human study protocol was reviewed and approved by the Ethic Committee of the University of Freiburg (decision number 22-1401-S1) and was performed with the 1964 Helsinki Declaration and its later amendments. Written informed consent was obtained from all individual participants before inclusion in the study.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Funding Sources

This work was supported in part by a grant from the Foundation for Sarcoidosis Research (Grant: DM 501-01) to M.A.F., G.Z., and P.E., Leonardis Stiftung (Grant: 03-2022) to M.A.F., and NC3Rs (Grant: NC/V001019/1) to G.F. We acknowledge the support of the Open Access Publication Fund of the University of Freiburg. The sponsors played no role in the design of the study.

Author Contributions

M.A.F., G.F., J.F., M.D.M., and W.S. designed the research, M.D.M., S.Z., P.E., C.K., H.S., L.H., A.H., J.F., G.F., P.Z., J.L., S.B., and M.A.F. performed the experiments. B.B., W.J.-D., Z.R., D.S., and S.M. contributed knockout animals, funding, and analytical tools. M.A.F., G.F., J.F., M.D.M., C.K., W.S., H.S., G.Z., P.N., and S.Z. analyzed the data. M.A.F., G.F., P.N., B.B., and W.S. wrote the manuscript.

Data Availability Statement

All data are available in the main text or in the supplement file containing online supplementary Figures S1–S6. Further inquiries can be directed to the corresponding author.

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