Expression of type I interferon by splenic macrophages suppresses adaptive immunity during sepsis

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Early during Gram-negative sepsis, excessive release of pro-inflammatory cytokines can cause septic shock that is often followed by a state of immune paralysis characterized by the failure to mount adaptive immunity towards secondary microbial infections. Especially, the early mechanisms responsible for such immune hypo-responsiveness are unclear. Here, we show that TLR4 is the key immune sensing receptor to initiate paralysis of T-cell immunity after bacterial sepsis. Downstream of TLR4, signalling through TRIF but not MyD88 impaired the development of specific T-cell immunity against secondary infections. We identified type I interferon (IFN) released from splenic macrophages as the critical factor causing T-cell immune paralysis. Early during sepsis, type I IFN acted selectively on dendritic cells (DCs) by impairing antigen presentation and secretion of pro-inflammatory

cytokines. Our results reveal a novel immune regulatory role for type I IFN in the initiation of septic immune paralysis, which is distinct from its well-known immune stimulatory effects. Moreover, we identify potential molecular targets for therapeutic intervention to overcome impairment of T-cell immunity after sepsis.

The EMBO Journal (2012) 31, 201–213. doi:10.1038/emboj.2011.380; Published online 28 October 2011

Subject Categories: immunology; molecular biology of disease
Keywords: dendritic cells; sepsis; splenic macrophages; T cells; type I IFN

Introduction

Sepsis is a severe inflammatory dysregulation arising during situations when infections are not confined locally but disseminate via the blood stream. The septic inflammatory response comprises two functionally distinct but not mutually exclusive phases: first, the systemic inflammatory response (SIRS) characterized by excessive production of pro-inflammatory mediators and second, the compensatory anti-inflammatory response (CARS). This later phase of sepsis is associated with increased susceptibility to secondary infections and is characterized by a severe impairment of adaptive T-cell immunity that subsequently can lead to an inability to control viral and bacterial infections, which may ultimately lead to multi-organ failure (Cohen, 2002; Hotchkiss and Karl, 2003; Hotchkiss *et al*, 2009). Various preclinical model systems have been developed to uncover pathophysiological cellular and molecular mechanisms responsible for SIRS and CARS, such as caecum ligation and puncture, colon ascendens stent peritonitis (CASP), injection of bacteria or Toll-like receptor (TLR) ligands such as LPS (Buras *et al*, 2005). Using these model systems, it became clear that both SIRS and CARS are initiated by widespread activation of innate immune cells as a consequence of signalling through pattern recognition receptors (PRRs) that detect pathogen-associated molecular patterns (PAMPs; Beutler, 2004; Kawai and Akira, 2010). While SIRS is associated with massive secretion of MyD88 and TRIF-dependent pro-inflammatory cytokines (i.e., TNF, interferon (IFN) γ , IL-1, IL-6; Weighardt *et al*, 2002; Weighardt and Holzmann, 2007), CARS is linked to the production of the regulatory cytokine IL-10 (Volk *et al*, 2000; Cohen, 2002), which is involved in dampening of innate and adaptive immune responses (Trinchieri, 2007; O'Garra and Murphy, 2009). IL-10 induces a refractory phase in macrophages that renders them non-responsive to PRR stimulation (Randow *et al*, 1995) and its serum levels correlate with decreased levels of MHC class II molecules and a defect in TNF production by monocytes of septic patients (Docke *et al*, 1997;

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Volk *et al*, 2000). Consequently, impairment of adaptive CD8⁺ T cell (CTL) immunity is considered to depend on an IL-10-mediated impairment of macrophages to initiate T-cell immunity.

Here, we show that type I IFN but not IL-10 exhibited an unexpected immune regulatory role for the generation of CTL immunity. Type I IFN produced locally in the spleen by macrophages in response to circulating bacteria incapacitated dendritic cell (DCs) to induce CTL immunity against subsequent systemic infections with viruses or intracellular bacteria.

Results

Systemic dissemination of bacteria prevents CTL immunity against systemic viral infections

Systemic distribution of bacteria plays an essential role in the induction of sepsis, SIRS and CARS. We used a well-established murine sepsis model CASP to investigate the role of bacterial translocation into the circulation for septic immune paralysis by intragastric application of recombinant luciferase-expressing *E. coli*, which allowed us to directly visualize the rapid bacterial spread after surgical intervention into various organs by *in-vivo* bioluminescence imaging (Figure 1A and B). Based on these results, we studied the mechanism determining immune regulation early during bacterial sepsis. To this end, we challenged mice surviving CASP with a systemic viral infection using recombinant adenovirus expressing Ovalbumin (AdOVA) and then determined the subsequent induction of OVA-specific T-cell immunity. We had shown recently that generation of adenovirus-induced CTL immunity strictly depends on antigen presentation by DCs in the spleen (Backer *et al*, 2010) and that systemic treatment of mice with the TLR-9-Ligand CpG-DNA resulted in inhibition of OVA-specific CTL immunity in the spleen (Wingender *et al*, 2006). We, therefore, reasoned that systemic distribution of bacteria would impair local induction of antiviral CTL immunity in the spleen. Quantification of the OVA-specific CTL response 5 days after AdOVA infection by measuring specific T-cell cytotoxicity *in vivo* revealed that antiviral CTL immunity was strongly suppressed in mice after CASP compared with untreated mice (Figure 1C). Further supporting our hypothesis that systemic bacteria suppress CTL responses in the spleen, mock-treated mice that had undergone only laparotomy and mechanical intestinal manipulation, but not CASP, showed bacterial translocation to liver and lung but only little bacterial dissemination into the spleen (Supplementary Figure S1). It has been reported previously that mechanical manipulation of the gut leads to inflammation of the intestinal wall causing translocation of gut bacteria (Schwarz *et al*, 2002). Consequently, as bacteria translocated to the liver and lung, but did not reach the spleen in significant numbers, those mice did not develop any impairment of CTL immunity (Figure 1C). As CTL immunity was also suppressed when using heat killed (HK) *E. coli* or when treating *E. coli*-infected mice with antibiotics (Supplementary Figure S2), we assume that the suppressive effect was independent of persistence of living bacteria. Furthermore, using defined numbers of *E. coli* instead of CASP had the advantage of investigating the effect of bacteria on generation of CTL responses in a quantitative manner. Thus, it was possible to show that systemic injected

E. coli suppressed generation of antiviral CTL immunity in a dose- and time-dependent fashion (Figure 1D). Furthermore, as i.v. injection of *E. coli* and CASP treatment suppressed CTL responses in a similar fashion we used i.v. injection of *E. coli* to mimic sepsis from now on throughout the manuscript.

Systemic distribution of *E. coli* not only suppressed development of CTL immunity against recombinant adenoviral antigens but also against Herpes Simplex virus (HSV), Influenza virus or *L. monocytogenes* infection (Figure 1E). As CTL immunity against these infectious microorganisms required the spleen (Figure 1F), our findings suggest that the immune paralysis observed after Gram-negative sepsis was a phenomenon affecting spleen-dependent CTL immunity in general. We recently showed that Adenovirus selectively infected metallophilic marginal zone macrophages (MMM). However, CD8⁺ T cells were not activated by MMM, but rather by CD8⁺DEC205⁺DCs, which received antigen from MMM by a so far undefined transfer mechanism (Backer *et al*, 2010). To exclude that during sepsis CTL responses against subsequent (bacterial or viral) infections were only impaired because due to a lower infectivity less antigen was available for presentation, infection with AdOVA was substituted by soluble OVA. Indeed, we could show that septic mice were also unable to mount a CTL response against soluble OVA (Figure 1G), thus confirming our hypothesis that sepsis had impeded CTL immunity by directly impairing T-cell activation, rather than by lowering antigen availability.

The inhibitory effect of bacterial sepsis was not restricted to CTL immunity, but extended to B-cell responses that required CD4 T-cell help. Systemic distribution of *E. coli* prior to immunization with soluble OVA plus stimulatory anti-CD40 antibody resulted in a significant lower titre of OVA-specific antibodies after 2 weeks (Supplementary Figure S3). Thus, we identified systemic bacterial distribution to the spleen as a critical event that suppressed development of antimicrobial CTL immunity as well as T helper cell-dependent antibody production.

Taken together, these data identified unique spatio-temporal characteristics of immune paralysis caused by Gram-negative bacteria to impair spleen-dependent T-cell immunity.

Suppression of CTL immunity induced by Gram-negative bacteria requires TLR4-dependent TRIF signalling

Circulating Gram-negative bacteria can be recognized by various PRRs such as the TLR 2 recognizing lipopeptides, TLR9 recognizing hypo-methylated bacterial DNA and TLR4 recognizing lipopolysaccharides. Whereas the contribution of TLRs to systemic inflammation after bacterial dissemination is well defined (Beutler, 2004), little is known about their role in inhibition of CTL immunity (Mellor *et al*, 2005; Wilson *et al*, 2006; Wingender *et al*, 2006). This led us to investigate whether individual TLR ligands inhibited CTL immunity. Similar to the inhibitory effect of *E. coli*, the TLR2 ligand Pam3Cys, the TLR4 ligand LPS and the TLR9 ligand CpG1668, all prevented induction of antiviral CTL immunity if applied intravenously 1 day before systemic viral infection (Figure 2A). While this suggested a broad involvement of several TLRs in regulation of

Figure 1 Systemic dissemination of bacteria prevented CTL immunity against systemic viral infections. (A, B) 10^{11} *E. coli* lux was given orally to C57BL/6 mice 1 h before CASP. Bacterial distribution measured and quantified by bioluminescence imaging at indicated time points *in vivo* or in isolated organs. (C, D) OVA-specific CTL immunity was determined *in vivo* in mice (C) that underwent CASP or (D) mice receiving different doses of *E. coli* or at different time points 1 day before i.v. challenge with AdOVA (5×10^9 particles/mouse). (E) i.v. injection of 10^6 *E. coli* 1 day prior to infection with Herpes Simplex Virus (HSV; 10^4 PFU), Influenza A virus (10^5 PFU) or *L. monocytogenes* (5×10^3). CTL immunity was determined 5 days later. (F) C57BL/6 mice were splenectomized or sham treated. Both groups of mice were i.v. infected with HSV, Influenza A or *L. monocytogenes* and 5 days later OVA-specific CTL immunity was determined *in vivo*. (G) Control, splenectomized and mice infected with 10^6 *E. coli* were immunized with soluble OVA plus anti-CD40 and CTL immunity was determined at day 5 in spleen. Representative data are shown as mean $\pm$ s.e.m. ($n = 4$ mice per group) of at least two independent experiments. (NS, not significant).

Figure 2 Suppression of CTL immunity induced by Gram-negative bacteria required TLR4-dependent TRIF signalling. (A) Mice were injected i.v. with LPS (1 µg), CpG-DNA (50 µg) or Pam3Cys (50 µg) 1 day before AdOVA infection and OVA-specific CTL immunity was determined. (B) I.v. injection of 10^6 *E. coli* into TLR2/4/9^{-/-}, (C), TLR2^{-/-}, TLR9^{-/-} or TLR4^{-/-} (D) MyD88^{-/-} or (E) TRIF^{-/-} mice 1 day before infection with AdOVA. OVA-specific CTL immunity was determined at day 5 in spleen. Data are shown as mean ± s.e.m. with $n = 3$ mice per group. Representative data of at least two independent experiments are shown (NS, not significant).

spleen-dependent T-cell immunity, we wondered whether these receptors were all required to induce immune paralysis *in vivo*. To this end, we evaluated induction of antiviral CTL immunity after bacterial dissemination in triple knockout mice that bear simultaneous deficiency for TLR2, 4 and 9. In these mice, we found no inhibition of CTL immunity by Gram-negative bacteria any more (Figure 2B). To identify the relevance of individual TLRs, we challenged single knockout mice intravenously with *E. coli* and then determined induction of antiviral CTL immunity. Surprisingly, only TLR4^{-/-} mice but neither TLR9^{-/-} nor TLR2^{-/-} mice mounted antiviral CTL immunity following Gram-negative sepsis (Figure 2C), demonstrating that during sepsis only TLR4 was crucial for inhibition of antimicrobial CTL immunity. The apparent discrepancy between the effect of TLR ligands and Gram-negative sepsis on development of immune paralysis may relate to distinct mechanisms employed by different cell populations to sense soluble TLR ligands or bacterial infection.

As TLR4 transmits signals via two adapter molecules, that is, MyD88 and TRIF, we next characterized the individual contribution of these adapter molecules to inhibition of antiviral CTL immunity by using mice deficient either for MyD88 or for TRIF. While Gram-negative sepsis still impaired CTL immunity in MyD88^{-/-} mice (Figure 2D), we observed generation of strong antiviral CTL immunity in TRIF^{-/-} mice (Figure 2E). These results identified a dominant and so far unknown function for TLR4 in inhibition of adaptive immunity in a TRIF-dependent fashion during the early phases of Gram-negative sepsis.

Critical role of type I IFN in *E. coli*-mediated inhibition of CTL immunity

Given the known regulatory function of IL-10 and its contribution to immune paralysis in patients (Docke *et al*, 1997) and given that activation through TLR2 and TLR9 impeded antiviral CTL immunity *in vivo* (Figure 2A), we first investigated the contribution of IL-10 in our model system. Unexpectedly, in IL-10^{-/-} mice, CTL immunity was still suppressed following infection with Gram-negative bacteria (Figure 3A) clearly demonstrating that IL-10 was not the reason for impaired development of CTL immunity.

To identify the factor that caused paralysis of adaptive immunity, we systematically investigated the events downstream of TRIF signalling. TRIF is known to potently cause induction of type I IFN expression (Beutler, 2004; Kawai and Akira, 2010). While the role of type I IFN on innate immunity early during sepsis is controversial, because it has been reported to promote (Karaghiosoff *et al*, 2003; Huys *et al*, 2009), or prevent (Kelly-Scumpia *et al*, 2010) pathology in different models of septic shock, the role of type I IFN on adaptive immunity during sepsis has not been investigated. We used mice deficient for the receptor of type I IFN (IFNAR^{-/-}) to characterize the relevance of type I IFN in regulating CTL immunity after bacterial sepsis. In these mice, we observed a modest induction of antiviral CTL immunity after Gram-negative sepsis that was increased compared with littermates but was still reduced compared with mice that were not subjected to Gram-negative sepsis (Figure 3B). This indicated a partial recovery from immune paralysis in the absence of type I IFN signalling. Interestingly, in contrast to

Figure 3 Critical role of type I IFN in *E. coli*-mediated inhibition of CTL immunity. I.v. injection of 10^6 *E. coli* into (A) IL-10^{-/-}, (B) IFNAR^{-/-}, (D) IRF3^{-/-}, IRF7^{-/-} or IRF3/7^{-/-} mice 1 day before i.v. challenge with AdOVA. (C) C57BL/6, IL-10^{-/-}, IFNAR^{-/-} and MyD88^{-/-} mice were either left untreated or received Pam3Cys or CpG-DNA 1 day prior to AdOVA infection. OVA-specific CTL immunity was determined at day 5 in spleen. Data are shown as mean $\pm$ s.e.m. with $n = 3$ mice per group. Representative data of at least two independent experiments are shown.

the clear dependence of *E. coli*-induced suppression on signalling via TRIF and IFNAR, suppression of CTL immunity by isolated TLR2 and TLR9 ligands was dependent on MyD88 and independent on signalling via IFNAR (Figure 3C). However, like *E. coli*-mediated suppression, suppression of CTL immunity by TLR2 and TLR9 ligands was independent of IL-10 (Figure 3C). For reasons not understood right now, although TLR2 and TLR9 ligands are expressed by *E. coli*, for CTL suppression only TLR4 ligands were functional.

In order to dissociate cell-intrinsic effects of TRIF-induced expression of type I IFN that is mediated directly by the IFN response factor 3 (IRF3), from autocrine/paracrine signalling via the IFNAR that is mediated by IRF7, we intravenously injected mice with *E. coli* followed by adenovirus. Compared with wild-type littermates we observed a partial recovery of CTL immunity for either knockout mouse line (Figure 3D). Consistent with the synergistic role of IRF3 and IRF7 in induction of type I IFN signalling (Honda *et al*, 2006), we observed also a synergistic effect of IRF3 and IRF7 to inhibit development of CTL immunity by using double knockout

mice (Figure 3D). Collectively, these results assign a novel role for type I IFN induced by TLR4/TRIF-dependent signalling in negative regulation of CTL immunity that is mediated via the synergistic activity of IRF3 and IRF7.

Type I IFN released from splenic macrophages blocks DC function to induce antimicrobial CTL immunity

These experimental results raised the question, which cell type was affected by type I IFN. To discriminate whether CTLs were directly incapacitated by type I IFN or whether expansion of CTLs by cross-priming DCs was affected, we first determined the numbers of OVA-specific CTLs in mice injected with Gram-negative bacteria before or after AdOVA infection. In mice suffering from sepsis prior to viral infection, suppression of CTL activity correlated with a severe reduction in expansion of virus-specific CTLs (Figure 4A). In contrast, in mice injected with *E. coli* after adenoviral infection CTL immunity was increased, correlating with a slightly increased virus-specific CTL expansion (Figure 4A). Based on these results, we speculated that type I IFN was the

cytokine, which had impaired DC functionality, such as cross-presentation and production of stimulatory cytokines. Indeed, pretreatment of mice with recombinant IFN α/β was able to suppress CTL immunity similar to *E. coli* (Figure 4B). Unlike injection of *E. coli* 1 day after AdOVA, injection of type I IFN 1 day after AdOVA did not significantly improve CTL

activity (Figure 4B). Thus, as this result clearly showed that type I IFN was suppressive at this point in time, we next addressed the question whether type I IFN had affected DC functionality. Indeed, CD11c⁺ cells isolated from the spleen of septic mice infected with AdOVA showed reduced cross-presentation of virus-derived OVA compared with DCs iso-

lated from mice not subjected to sepsis (Figure 4C). Interestingly, DCs isolated from septic and non-septic mice revealed no differences in absolute numbers of cross-presenting CD8⁺ DCs and even upregulated CD86, MHC-I and MHC-II molecules that are essential for T-cell activation (Supplementary Figure S4). These results imply that systemic bacteria had neither deleted DCs nor downregulated MHC molecules needed for antigen presentation. Besides affecting cross-presentation, type I IFN had also impaired IL-12p70 and IL-6 production detected *ex vivo* in splenic DCs (Figure 4D) demonstrating that type I IFN induced by *E. coli* severely impaired DC functionality. Furthermore, in support of our *in-vivo* data and data from the literature (Longman *et al*, 2007), pretreatment of BM-DCs with type I IFN also led to a reduction in cross-priming of naive CD8 T cells *in vitro* (Figure 4E). Similar to our *ex-vivo* data, antigen uptake and MHC I and II expression were not affected by type I IFN (Supplementary Figure S5).

To confirm this immune regulatory role of type I IFN on DCs and to exclude a direct suppressive effect on T cells, we studied whether CTL immunity developed after challenge with *E. coli* in mice lacking IFNAR selectively on CD11c⁺ cells or T cells. Interestingly, in septic mice lacking IFNAR on DCs, but not in mice lacking IFNAR on T cells, a normal antiviral CTL response was generated (Figure 4F). This result demonstrates that DCs were a key target cell population for the immune regulatory function of type I IFN.

As CD4 T-cell help was critical for induction of adenovirus-specific CTL immunity (Supplementary Figure S6) and as *E. coli* injection impaired the generation of CD4 help-dependent IgG production (Supplementary Figure S2), we next investigated the role of CD4 T cells in sepsis-induced paralysis of CTL immunity. Importantly, sepsis-induced inhibition of CTL immunity was prevented *in vivo* through pre-existent CD4 T-cell help generated by prior vaccination of mice with E15R, an MHC class II binding OVA peptide known to induce a strong CD4 T-cell response (Figure 4G; Bonifaz *et al*, 2002; Boscardin *et al*, 2006). In order to differentiate whether CD4 T-cell help acted directly on DCs to overcome the inhibitory effects of type I IFN, we targeted antigen directly to DCs using DEC205-OVA in combination with anti-CD40 antibodies, that are known to functionally mature DCs by mimicking CD4 T-cell help (Bonifaz *et al*, 2002; Bevan, 2004; Boscardin *et al*, 2006; Kurts *et al*, 2010). Although anti-CD40 treatment efficiently induced CTL immunity in untreated mice indicating functional maturation of DCs, such treatment did not prevent inhibition of antiviral CTL immunity after sepsis (Figure 4H).

This indicated that DCs were severely impaired by type I IFN in their function to stimulate T cells. Moreover, we observed a reduced MHC-II restricted presentation to naive CD4 T cells after treatment of immature DCs with type I IFN *in vitro* (Figure 4I), suggesting that type I IFN not only rendered DCs non-responsive to CD4 T cell-mediated maturation but also already impaired generation of CD4 T-cell help.

Type I IFN produced by splenic macrophages selectively impairs DCs

As IRF3 and IRF7 acted synergistically to inhibit CTL immunity (Figure 3D) inhibition of the DC function to generate CTL immunity likely is the consequence of a combination of direct TLR4/TRIF-dependent IRF3 signalling and IRF7 signalling triggered by binding of type I IFN to the IFNAR on DCs. In combination with complete recovery of function in DCs lacking IFNAR expression this suggested that type I IFN produced by other cell types in response to TLR4/TRIF signalling might be acting on DCs. To identify this cell population producing type I IFN, we examined the cellular distribution in the spleen of systemically circulating recombinant bacteria that gain fluorescence activity after phagocytic uptake. Such bacteria were found in the marginal zone of the spleen in the vicinity of Siglec1⁺ macrophages (Figure 5A). Local expression of type I IFN within the marginal zone in response to bacteria was suggested by strong expression of the IFN-sensitive MxA protein (Figure 5B) and was directly detected by measuring type I IFN gene expression by RT-PCR (Figure 5C). Expression levels of type I IFN in the spleen (Figure 5C, left panel) directly correlated with inhibition of CTL immunity (Figure 5C, right panel).

To directly characterize the relevance of phagocytic cells in type I IFN-mediated immune paralysis, we depleted macrophages with clodronate liposomes and waited for 7 days, which allows CD11c⁺ DC to recover while depletion of macrophages persists (Backer *et al*, 2010), before injecting mice intravenously with *E. coli*. Interestingly, clodronate treatment abrogated sepsis-induced expression of type I IFN in the spleen (Figure 5D), suggesting that macrophages were instrumental in suppression of T-cell immunity. Consequently, in the absence of macrophages, DCs targeted by DEC205-OVA were then able to mount antiviral CTL immunity after sepsis (Figure 5E). The importance of macrophages for suppression of CTL immunity was further demonstrated in experiments using mice lacking IFNAR only on macrophages (LysMCre × IFNAR^{fl/fl}). Similarly to mice lacking IFNAR on DCs, CTL responses in septic mice lacking

Figure 4 Type I IFN blocked DC ability to induce antimicrobial CTL immunity. (A) C57BL/6 mice received 10⁶ *E. coli* i.v. either 1 day before or 1 day after infection with AdOVA; 5 days later cytotoxic activity (right panel) was correlated with frequencies of OVA-specific CTLs, which were determined by S8L/H-2K^b pentamer staining (left panel). (B) C57BL/6 mice received 10⁶ *E. coli* or 1000 IU IFN α / β i.v. 1 day before or IFN α / β 1 day after infection with AdOVA. Cytotoxic response was determined 5 days later in spleen. (C) CD11c⁺ cells were isolated from spleens 24 h after AdOVA infection from control mice and mice pretreated with 1 × 10⁶ *E. coli*. CD11c⁺ cells were co-incubated o/n with OTI T cells and supernatants were analysed for IL-2 by ELISA. (D) DCs of control mice or mice injected with 10⁶ *E. coli* or IFN α / β were isolated 24 h later from spleen and restimulated with CpG-DNA *in vitro*. Supernatants were analysed for IL-6 and IL-12 24 h later by ELISA. (E) Immature BM-DCs were treated with 300 units/ml IFN α / β for 16 h, followed by incubation with indicated concentrations of OVA for 3 h. T-cell activation was determined by IL-2 ELISA from supernatant after o/n incubation of DCs with (E) OT-I or (I) OT-II cells. (F) OVA-specific CTL immunity was determined in C57BL/6 and CD11cCre IFNAR^{fl/fl} mice. Mice were either left untreated or injected i.v. with 10⁶ *E. coli* 1 day prior to infection with AdOVA. (G) Mice were either immunized s.c. with OVA derived I-Ab peptide E15R and pIC/CpG-DNA or left untreated. Seven days later, indicated mice were injected i.v. with 10⁶ *E. coli* and infected with AdOVA 24 h later; OVA-specific CTL immunity was determined 5 days later. (H) One day before mice received a combination of 5 μ g anti-DEC205-OVA i.v. + 20 μ g anti-CD40, mice were injected i.v. with 10⁶ *E. coli* or left untreated. Five days later OVA-specific CTL immunity was determined in spleen. Data are shown as mean $\pm$ s.e.m. with *n* = 3 mice per group. Representative data of at least two independent experiments are shown.

Figure 5 Type I IFN by splenic macrophages impairs DC function during sepsis. (A) 200 μ l of pHrodo *E. coli* Bioparticles were administered i.v. in wt mice and cryo sections of the spleen were prepared 1 h after infection. Siglec1 on metallophillic marginal zone macrophages (MMM) was stained with Ser-4 (green), pHrodo *E. coli* bioparticles (red). (B) C57BL/6 mice or mice expressing the MxA protein were infected with 10^6 *E. coli* i.v. 1 day after bacterial infection MxA protein was detected by immunohistochemistry. One mouse out of three for each group is depicted. Original magnification $\times 200$ and $\times 400$ (small panel). (C) C57BL/6 mice were infected with increasing doses of *E. coli* (10^3 – 10^6) or injected with PBS. In half of the mice, relative expression of IFN β 1 was measured by quantitative PCR in spleen tissue 6 h later (left panel), while the other half was infected with AdOVA 24 h later and CTL immunity was determined in spleen on day 5 (right panel). (D) 7 days before injection with 10^6 *E. coli*, mice were depleted of macrophages by clodronate liposome or injected with PBS; relative expression of IFN β 1 was measured by quantitative PCR in spleen tissue 6 h later. (E) Macrophages of wt mice were depleted by clodronate liposomes (200 μ l i.v.). Seven days after depletion mice were infected i.v. with 10^6 *E. coli* or left untreated. One day after infection mice were immunized i.v. with 3 μ g anti-DEC205-OVA + 20 μ g anti-CD40 and OVA-specific CTL immunity was determined in spleen 5 days after immunization. (F) OVA-specific CTL immunity was determined in C57BL/6 and LysMCre IFNAR^{fl/fl} mice. Mice were either left untreated or injected i.v. with 10^6 *E. coli* 1 day prior to infection with AdOVA. (G) C57BL/6, CD11cCre IFNAR^{fl/fl}, LysMCre IFNAR^{fl/fl} or IFNAR^{-/-} mice were either left untreated or received 10^6 *E. coli* i.v. Six hours later, relative expression of IFN β 1 was measured by quantitative PCR in spleen tissue. Data are normalized to the arithmetic mean of all ΔC_t IFN β 1 values from all wild-type mice. Data are shown as mean $\pm$ s.e.m. with $n = 3$ mice per group (NS, not significant).

IFNAR on macrophages were no longer suppressed (Figure 5F). These results demonstrate that macrophages not only control CTL immunity by transferring antigen to CD8⁺ DCs (Backer *et al.*, 2010), but also regulate T-cell activation via type I IFN. Figure 5D clearly shows that macrophages were responsible for the production of type I IFN after interaction with bacteria. Only little type I IFN expression was detected when IFNAR expression was absent from DCs, macrophages or all cells (Figure 5G). This is most likely explained by amplifying positive feed forward loop in macrophages and DCs to increase initial IFN responses.

Taken together, our findings support the notion that during sepsis both macrophages and DCs contribute to amplification of type I IFN expression that in turn negatively regulates the ability of DCs to mount CTL immunity.

Discussion

Bacterial sepsis is a severe complication of systemic infection, which can be divided into two phases: the first phase is characterized by an initial overwhelming inflammatory response and a second phase, which is characterized by the

inability to mount protective adaptive immune responses against secondary microbial infections. Here, we show that following Gram-negative sepsis mice develop an immune paralysis and fail to mount CTL immunity against viral (Adenovirus, HSV, Influenza A virus) and bacterial infections (*L. monocytogenes*). Such septic immune paralysis depended on TLR4-selective recognition of bacteria and TRIF-mediated signalling via IRF-3/7 to induce type I IFN in macrophages, which directly incapacitated DCs to present antigen and activate CD8 T cells.

We have recently identified the spleen to be critical for the induction of adaptive T-cell immunity against systemic viral infections (Backer *et al*, 2010). We have shown that in the spleen CD8 T-cell activation by CD8⁺ DCs was strictly dependent on antigen transfer from MDM to CD8⁺ DCs, an until then unknown mechanism operating in the splenic marginal zone (Backer *et al*, 2010). Here, we report that septic paralysis of T-cell immunity was only observed when bacteria disseminated in sufficiently high numbers via the blood stream into the spleen, indicating that suppressive mechanisms acted mostly in the spleen. This hypothesis is further supported by reports showing that the spleen is centrally affected during bacterial sepsis (Cohen, 2002; Hotchkiss and Karl, 2003; Hotchkiss *et al*, 2009). So far, immune paralysis was described to result from irreversible changes in the splenic architecture and depletion of immune cells occurring as a late consequence of bacterial sepsis (Cohen, 2002; Hotchkiss and Karl, 2003; Hotchkiss *et al*, 2009). Our results demonstrate, however, that at least at early time points after SIRS immune paralysis is a reversible state not yet caused by cell depletion but by functional inhibition of DCs.

Mechanistically, we identified macrophages as the key cell population sensing systemic bacteria and suppressing DC function by secreting type I IFN and thereby preventing generation of T-cell immunity. Type I IFN was initially produced by macrophages in the marginal zone of the spleen. As in the absence of IFNAR on either cell population *E. coli* induced much less type I IFN, we concluded that the initial weak type I IFN response was amplified by a concert action of both macrophages and DCs.

This new regulatory function of macrophages nicely adds to the already known functions of macrophages, i.e., when macrophages are activated in the context of a microbial infection they undergo two phases: initially, they stimulate immunity through production of pro-inflammatory mediators such as TNF and at later time points inhibit innate and adaptive immunity by expression of IL-10. Consequently, in patients suffering from sepsis increased levels of IL-10 were measured in serum, which was directly correlated with the observed impaired adaptive immunity. Furthermore, it has been convincingly shown that monocytes of such septic patients exhibit a decreased expression of MHC II molecules and an inability to produce TNF (Docke *et al*, 1997; Volk *et al*, 2000). Despite this clear association of septic immune paralysis with IL-10, immune paralysis, as observed here at early time points after sepsis, clearly did not depend on IL-10 but on type I IFN. This suggests that distinct phases of septic immune paralysis exist, which seem to be governed by different molecular mechanisms: An early phase that is regulated by type I IFN causing a reversible suppression of adaptive immunity and (later) phases, which are characterized by deletion of immune cells and overall irreversible

changes in the spleen (Cohen, 2002; Hotchkiss and Karl, 2003; Hotchkiss *et al*, 2009), involving IL-10 at some point.

How do macrophages sense Gram-negative bacteria? To our surprise, only TLR4 was instrumental in sensing bacterial infection leading to early septic immune paralysis, although Gram-negative bacteria in principle should be sensed by a variety of TLRs recognizing other structural components. In this respect, death from Gram-negative septic shock induced by a massive bacterial load was reported to also depend exclusively on TLR4 (Roger *et al*, 2009), indicating that this receptor is essential for both phases of sepsis, the inflammatory and the immune paralysis phase. However, while death during the inflammatory phase involved both adapter molecules downstream of TLR4, that is, MyD88 and TRIF (Weighardt *et al*, 2002, 2004), immune paralysis during early septic immune paralysis occurred only in response to TRIF-mediated but not to MyD88-mediated signalling. This again is consistent with type I IFN being the regulatory mediator of this suppression.

Type I IFN has pleiotrophic effects and has been reported to promote pro-inflammatory as well as anti-inflammatory responses (Biron, 2001; Trinchieri, 2010). Among others, the complexity of type I IFN activity is demonstrated by its clinical use to either attenuate autoimmunity in multiple sclerosis or to increase T-cell immunity in chronic hepatitis B and C (Trinchieri, 2010). Without any doubt, type I IFN is a strong activator of adaptive immunity, by stimulating both DCs and T cells (Biron, 2001; Curtsinger *et al*, 2005; Longhi *et al*, 2009; Trinchieri, 2010). Among the many reports describing an immune stimulatory role for type I IFN some reports clearly demonstrate that type I IFN stimulates CTL immunity by enhancing DC differentiation and maturation (Hoebe *et al*, 2003; Honda *et al*, 2003) improving cross-priming (Le Bon *et al*, 2003; Dunn *et al*, 2005) and by directly stimulating T-cell proliferation as signal 3 (Curtsinger *et al*, 2005). On the other hand, type I IFN has been shown to inhibit antiviral immunity by blocking DC differentiation from precursor cells (Hahm *et al*, 2005), by inhibiting cross-presentation *in vitro* (Longman *et al*, 2007) and by inducing a refractory state in DCs (Zuniga *et al*, 2008). Adding to this list of reports ascribing a negative regulatory role for type IFN, we report here a novel negative regulatory function for type I IFN in inhibiting DC function early after Gram-negative sepsis where type I IFN jeopardized antiviral as well as antibacterial CTL immunity. How can the paradox of type I IFN being both stimulatory and suppressive be explained? Our observations suggest that type I IFN has distinct effects depending on the differentiation status of the DC and time point and location of IFN production. In this respect, we have observed that systemic *E. coli* was only suppressive if injected before AdOVA infection, and was rather stimulatory, if injected after AdOVA infection. Furthermore, the suppressive effect of *E. coli* could be replaced by recombinant type I IFN, proving its suppressive potential when given before antigen challenge. In line with our observations, also other groups have reported paradox effects of type I IFN (Hahm *et al*, 2005; Longman *et al*, 2007). The authors explain this seemingly contradicting effect of type I IFN by correlating stimulatory and suppressive functions of type I IFN with the usage of different STAT molecules downstream of the IFNAR (Hahm *et al*, 2005; Longman *et al*, 2007). They convincingly show that immature DCs, where IFNAR is associated with STAT1

and STAT3, are suppressed, while matured DCs, where IFNAR is associated with STAT4, are further activated by type I IFN. These data explain how the same cytokine can have opposing effects. As immature DCs are situated at the splenic marginal zone (Idoyaga *et al*, 2009), it is conceivable that during sepsis type I IFN produced by marginal zone macrophages strongly suppresses those immature marginal zone DCs, while activated DCs, which already have migrated to the T-cell zone, are stimulated by type I IFN.

While stimulatory and inhibitory mediators secreted by macrophages allow clear distinction of signals at the molecular level, it is interesting to speculate why a dual function for type I IFN during an immune response has evolved. The spleen has a dual role mounting immunity to microbial infection and at the same time in eliminating dying senescent cells and cellular debris from the blood circulation. Tissue damage and cell death can cause the release of endogenous danger signals such as HMGB1 that cause sterile inflammation by signalling through RAGE and TLR4 (Sims *et al*, 2010; Yang *et al*, 2010). Massive systemic inflammation might eventually trigger autoimmunity by activating auto-reactive T cells via DCs presenting autoantigens.

A possible explanation for the seemingly paradox role of type I IFN on adaptive immunity might be related to the time point when type I IFN acts on antigen-presenting cells: while there is ample evidence that the presence of type I IFN during antigen presentation to T cells leads to immunity, our data suggest that temporal dissociation of type I IFN production from antigen presentation can result in inhibition of DC function. If our reasoning is correct, it should allow the immune system to differentiate between dangerous pathogens and self-proteins, simply because only pathogens would provide both signals simultaneously, that is, antigen and PAMPs, whereas self-antigens do not. The hypothesis of loco-temporal differentiation of self and non-self is supported by a report, which convincingly showed that T-cell activation was already prevented, if antigen and TLR ligands were present in the same APC, but separated in different vesicles (Blander and Medzhitov, 2006). Thus, we speculate that the mechanism reported here, has evolved as fail-safe mechanism to protect from autoimmunity during infection. Therefore, the development of autoimmunity in some individuals following infections may be related to a failure of type I IFN-mediated control of DC function. With respect to sepsis, induction of immune paralysis via TLR4 may represent an accidental activation of this fail-safe mechanism by systemic bacteria expressing molecular structures resembling endogenous TLR4 ligands (Cohen, 2002; Hotchkiss and Karl, 2003; Hotchkiss *et al*, 2009).

Finally, since type I IFN also has deleterious effects during the early inflammatory phase of sepsis (Karaghiosoff *et al*, 2003; Weighardt *et al*, 2006) in addition to macrophage-derived type I IFN being the key mediator in CTL immune paralysis after sepsis, cell type-specific knockdown strategies targeting TLR4 and/or TRIF-induced type I IFN expression in macrophages may be exploited to improve the immune status in patients suffering from sepsis.

Materials and methods

Animals

In all, 6–8-week-old C57BL/6 mice were purchased from Janvier, France. Experiments were performed using wild-type C57BL/6

mice, MxA transgenic mice (Wenzel *et al*, 2005) and mice deficient for TLR2 (Takeuchi *et al*, 1999), TLR4 (Hoshino *et al*, 1999), TLR9 (Hemmi *et al*, 2000), TLR2/4/9, TRIF (Yamamoto *et al*, 2003), MyD88 (Adachi *et al*, 1998), IL-10 (Kuhn *et al*, 1993), IRF3 (Sato *et al*, 2000), IRF7 (Honda *et al*, 2005), IRF3/7 (Steinberg *et al*, 2009), IFNAR (van den Broek *et al*, 1995), I-Ab (Cosgrove *et al*, 1991) or deficient for IFNAR selectively on CD11c⁺ cells (CD11cCre IFNAR^{fl/fl}) or macrophages (LysMCre IFNAR^{fl/fl}) (Cervantes-Barragan *et al*, 2009). All mice had been bred on the C57BL/6 background in accordance with institutional animal guidelines under specific pathogen-free conditions in the local animal facility (House for Experimental Therapy, HET) of the University of Bonn. Mouse studies were approved by the local regulatory agencies (LANUV NRW §9.93.2.10.35.07.268 and §9.93.2.10.35.07.269).

Reagents

RPMI medium (Sigma, Munich, Germany) supplemented with 10% FCS (PAA, Cölbe, Germany), 1% penicillin/streptomycin (Lonza, Cologne, Germany), 1% L-glutamine (PAA) and 50 µM β-mercaptoethanol (Sigma) was used for DC/T-cell co-cultures. Peptides (Pineda, Germany) were dissolved as 20 mM stock solutions in DMSO and stored at –20°C. TLR-L: CpG-ODN1668 (TIB MolBio, Berlin), Pam3Cys and ultrapure LPS (InVivoGen, France) and pIC (Sigma). IFNα/β was purchased from R&D. To deplete macrophages, mice received 200 µl of clodronate liposomes i.v. at indicated time points.

Surgical procedures

The CASP procedure used for induction of septic peritonitis was described in detail previously (Zantl *et al*, 1998). Here, we used a 20-gauge venous catheter that was inserted into the colon ascendens ~10 mm distal of the ileocaecal valve. For mock treatment, the small bowel of the animals was subjected to a standardized surgical manipulation (IM), that is, intestines of mice were only touched with cotton swabs without setting a stent therefore mimicking the complex surgical procedure during CASP treatment (Schwarz *et al*, 2002). For splenectomy, anaesthetised mice underwent laparotomy and the spleen was removed after blood vessel ligation. Mice were used 1 week after splenectomy for experiments.

Experimental infections and immunizations

Escherichia coli expressing the lux operon (*E. coli* lux), wild-type *E. coli* or recombinant *Listeria monocytogenes* expressing OVA (LmOVA) was cultured for 4 h in Luria-Bertani broth (Roth, Karlsruhe) containing ampicillin (20 mg/l) or in brain heart infusion broth medium (Roth). For *in-vivo* killing of *E. coli*, ampicillin (20 mg/mouse) was injected i.p. 1 h after *E. coli* injection. Colony forming units (CFUs) were quantified by determining optical density at 600 nm. HK *E. coli* was generated by 60 min incubation at 70°C. Pathogens and other reagents were generally diluted in 0.9% PBS for experimental infections. For experimental viral infections, mice were infected i.v. with 5×10^9 virus particles of recombinant, replication incompetent Adenovirus (Ad5) expressing ovalbumin (AdOVA) (Schweichel *et al*, 2006), 10^4 PFU HSV type 1 or 10^5 PFU Influenza A PR8/H1N1 virus. For induction of OVA-specific cytotoxicity, mice were either infected i.v. with AdOVA or injected i.v. with (3 µg/mouse) anti-DEC205-OVA or (50 µg/mouse) soluble OVA (Sigma, grade V) in combination with (20 µg/mouse) anti-CD40 antibody. For induction of OVA-specific CD4 cells, endogenous OVA-specific CD4 T cells were activated *in vivo* with 50 µg OVA_{265–279} (E15R) (Boscardin *et al*, 2006) mixed with 5 µg CpG-1668 and 5 µg pI:C by subcutaneous (s.c) immunization into the flank 6 days before adenovirus infection. Application routes (volume) are intravenous (200 µl) and subcutaneous (100 µl).

Determination of bacterial uptake and in-vivo distribution

For *in-vivo* imaging, mice received 10^{11} *E. coli* lux per gavage and were analysed under inhalational anaesthesia with isoflurane using the Living Image 2.50 software[®]. A single cell suspension was prepared from organs by pressing the organs through a sterile sieve (Ø 250 µm). In all, 100 µl of the cell suspension was plated on selective MacConkey Agar and after 24 h CFUs were calculated per organ and per gram tissue, respectively.

pHrodoTM *E. coli* bioparticles were injected i.v. into mice and the spleen was removed 1 h later for histological analysis. Spleens were fixed for 30 min in 1% PFA and transferred into PBS containing 30%

(w/v) sucrose. Spleens were briefly washed with PBS and embedded in O.C.T.TM medium and frozen at -80°C . Following incubation of sections ($5\mu\text{m}$) with aSER-4 and aCD11c mAbs (45 min), sections were stained with anti-GFP mAb and Streptavidin-AlexaFluor[®]488 (30 min). Nuclei were stained with $1\mu\text{g}/\text{ml}$ DAPI for 10 min. MxAb labelling was performed on paraffin-embedded tissue sections (4mm). Sections were incubated with anti-MxAb mAb (dilution 1:100) overnight at room temperature. Secondary labelling was performed using the LSAB2 kit (DAKO) (Wenzel *et al*, 2005).

Cell isolation, ex-vivo co-culture, cell culture assays

BM-DCs were generated as described before (Busch *et al*, 2008). For antigen presentation assays, BM-DCs were incubated with 2×10^5 OT-I or OT-II cells. T-cell activation was determined by measuring IL-2 by ELISA. CD8a⁺ T cells were obtained from spleens and lymph nodes of OT-I/RAG1^{-/-} transgenic mice (Hogquist *et al*, 1994) and enriched using nylon wool (Kisker Biotech, Steinfurt, Germany). For isolation of DCs *ex vivo*, spleens were collagenase digested for 15 min before isolating cells. After incubation with anti-CD11c magnetic beads (Miltenyi), DCs were purified using auto-MACS[®] (Miltenyi). 3×10^5 purified CD11c⁺ DCs were co-cultured with 10^5 naive OVA-specific CD8 T cells in flat-bottom 96-well plates. Co-culture supernatants for ELISA were collected after 24 h. To determine the effect of systemic bacteria on cross-presentation, 1×10^5 CD11c⁺ DCs were isolated from spleens of control mice, and mice infected with 1×10^6 *E. coli* prior to infection with AdOVA. DCs were incubated with 2×10^5 isolated OTI cells and IL-2 secretion was determined after o/n incubation by ELISA. To determine IL-6 and IL-12 production of *ex-vivo* isolated DCs, DCs were isolated as described above and stimulated with $0.5\text{ ng}/\text{ml}$ CpG-DNA. After 24 h, supernatants were analysed by ELISA.

Flow cytometry

For flow cytometric analysis, cells were stained with fluorochrome-conjugated anti-mouse CD8a, CD11b, CD11c, CD86, I-A/I-E, H2-K^b (eBioscience, San Diego, USA or BD Biosciences, Heidelberg, Germany), or H-2K^b/SIINFEKL pentamers (S8L/H-2K^b; Proimmune, Oxford, UK). Hoechst-33258 ($1\mu\text{g}/\text{ml}$) was added to exclude dead cells and cells were analysed by flow cytometry (BD Canto II). For detection of OVA-specific CD8 T cells, splenocytes of each group were pooled and stained with anti-CD8a and S8L/H-2K^b pentamers according to manufacturers guide. Absolute cell numbers were determined with calibrite beads[®] (BD Bioscience). For analysis of antigen uptake, cells were incubated with $0.5\mu\text{g}/\text{ml}$ OVA-Alexa647 (Invitrogen, Darmstadt, Germany) for 15 min and analysed by flow cytometry.

CTL activity was determined *in vivo* as described previously (Feuerer *et al*, 2003; Wingender *et al*, 2006). Briefly, splenocytes from syngeneic donor mice were pulsed with $1\mu\text{M}$ of the specific H-2K^b peptides SIINFEKL (Ovalbumin), ASNENMETM and SLENFRAYV (Influenza A PR8), or SSIEFARL (HSV), for 30 min at 37°C and subsequently with $1\mu\text{M}$ of CFSE or FarRed for 15 min (CFSE^{high} or FarRed, specific target cells). Reference cells were not pulsed with peptide and labelled with $0.1\mu\text{M}$ CFSE for 15 min (CFSE^{low}, reference cells). Both cell populations were mixed at a 1:1 ratio (CFSE^{high}/FarRed:CFSE^{low}) and 10^7 total cells were injected into the tail vein. Four hours later, lysis of peptide-loaded cells was quantified by measuring the ratio of CFSE^{high}/FarRed and CFSE^{low} cells via flow cytometry (Canto II, BD Biosciences). The percentage of specific lysis was calculated using the following equation: $100 - [(CFSE^{\text{high}}/CFSE^{\text{low}})_{\text{immunized}} / (CFSE^{\text{high}}/CFSE^{\text{low}})_{\text{naive}}] \times 100$.

References

- Adachi O, Kawai T, Takeda K, Matsumoto M, Tsutsui H, Sakagami M, Nakanishi K, Akira S (1998) Targeted disruption of the MyD88 gene results in loss of IL-1- and IL-18-mediated function. *Immunity* **9**: 143–150
- Backer R, Schwandt T, Greuter M, Oosting M, Jungerkes F, Tuting T, Boon L, O'Toole T, Kraal G, Limmer A, den Haan JM (2010) Effective collaboration between marginal metallophilic macrophages and CD8⁺ dendritic cells in the generation of cytotoxic T cells. *Proc Natl Acad Sci USA* **107**: 216–221
- Beutler B (2004) Inferences, questions and possibilities in Toll-like receptor signalling. *Nature* **430**: 257–263

Analysis of cytokine production

IL-2, IL-6, IFN γ and IL-12 in serum and supernatants were determined using standard sandwich ELISA according to the manufacturer's guide (eBiosciences, San Diego, USA).

Groups of three mice were infected with *E. coli* at different doses (1×10^3 , 1×10^4 , 1×10^5 , 1×10^6 ($n = 3$)). To test the influence of macrophages in these setting additional three mice were treated with clodronate liposomes ($200\mu\text{l}$ i.v.) at day -7 prior infection with *E. coli* (1×10^6). Fresh frozen spleen samples from all case groups as well as from three wild-type mice were pestled in liquid nitrogen and subsequently lysed in Trizol (Invitrogen). In all, 500 ng RNA was reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Penzberg, Germany). RT-PCR was performed using the Light Cycler Taqman master kit thereby amplifying the IFNB1 gene (NM_010510.0) and the 18S rRNA gene as reference control based on a Universal ProbeLibrary Assay on a Light Cycler 480 instrument (Roche). Analysis was performed with the Light-Cycler 480 Software release 1.5.0 and RelQuant software (Roche). Briefly, the IFNB1/18S ratio was calculated for each of the samples using the ΔC_t method. Next, data were normalized by dividing the ΔC_t values for IFNB1 of each groups of through the arithmetic mean of all ΔC_t IFNB1 values from the wild-type mice ($\Delta\Delta C_t$ value). Data were analysed by applying a one-sided unpaired *T*-test assuming different variances.

Primers used were m18sRNAforward#55: AAATCAAGTTATGGT TCCTTGTC, m18sRNAreverse#55: GGCTAGGAAATTACCACAGT TATCCAA, internal probe 55. mIFNB1forward#18: CTGGCTCCA TCATGAACAA, mIFNB1reverse#18: AGAGGCTGTGGTGGAGGAA, internal probe 18.

Software and statistics

Microscopy Acquisition Software Cell F (Olympus). Results are depicted as mean $\pm$ standard error of the mean (s.e.m.). Statistical significance was calculated by an unpaired two-tailed Student's *t*-test using the Prism[®] software. *P*-values of 0.01–0.05 were considered as significant (*), *P*-values of 0.001–0.01 as highly significant (**) and *P* of <0.001 as extremely significant (***).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

Acknowledgements

We thank the flow cytometry core facility, the house for experimental therapy (HET) and Michael Kraut for excellent technical assistance. The study was funded by grants of the DFG (KFO115 and SFB704) to AL.

Author contributions: TS and AL designed research. TS, BS, FJ, GHG, PS, KK, AS-J, IL-P, JW, RZ and SB performed experiments. NvR, GK, SW, JCK, JMMdh, CK, CC, UK, CK, RZ, BH, LL and JLS contributed new reagents or analytic tools. TS, BS, FJ, GHG, JLS and AL analysed the data. TS, PK and AL wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

- Bevan MJ (2004) Helping the CD8(+) T-cell response. *Nat Rev Immunol* **4**: 595–602
- Biron CA (2001) Interferons alpha and beta as immune regulators—a new look. *Immunity* **14**: 661–664
- Blander JM, Medzhitov R (2006) Toll-dependent selection of microbial antigens for presentation by dendritic cells. *Nature* **440**: 808–812
- Bonifaz L, Bonnyay D, Mahnke K, Rivera M, Nussenzweig MC, Steinman RM (2002) Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I pro-

- ducts and peripheral CD8⁺ T cell tolerance. *J Exp Med* **196**: 1627–1638
- Boscardin SB, Hafalla JC, Masilamani RF, Kamphorst AO, Zebroski HA, Rai U, Morrot A, Zavala F, Steinman RM, Nussenzweig RS, Nussenzweig MC (2006) Antigen targeting to dendritic cells elicits long-lived T cell help for antibody responses. *J Exp Med* **203**: 599–606
- Buras JA, Holzmann B, Sitkovsky M (2005) Animal models of sepsis: setting the stage. *Nat Rev Drug Discov* **4**: 854–865
- Busch A, Quast T, Keller S, Kolanus W, Knolle P, Altevogt P, Limmer A (2008) Transfer of T cell surface molecules to dendritic cells upon CD4⁺ T cell priming involves two distinct mechanisms. *J Immunol* **181**: 3965–3973
- Cervantes-Barragan L, Kalinke U, Zust R, Konig M, Reizis B, Lopez-Macias C, Thiel V, Ludewig B (2009) Type I IFN-mediated protection of macrophages and dendritic cells secures control of murine coronavirus infection. *J Immunol* **182**: 1099–1106
- Cohen J (2002) The immunopathogenesis of sepsis. *Nature* **420**: 885–891
- Cosgrove D, Gray D, Dierich A, Kaufman J, Lemeur M, Benoist C, Mathis D (1991) Mice lacking MHC class II molecules. *Cell* **66**: 1051–1066
- Curtsinger JM, Valenzuela JO, Agarwal P, Lins D, Mescher MF (2005) Type I IFNs provide a third signal to CD8⁺ T cells to stimulate clonal expansion and differentiation. *J Immunol* **174**: 4465–4469
- Docke WD, Randow F, Syrbe U, Krausch D, Asadullah K, Reinke P, Volk HD, Kox W (1997) Monocyte deactivation in septic patients: restoration by IFN- γ treatment. *Nat Med* **3**: 678–681
- Dunn GP, Bruce AT, Sheehan KC, Shankaran V, Uppaluri R, Bui JD, Diamond MS, Koebel CM, Arthur C, White JM, Schreiber RD (2005) A critical function for type I interferons in cancer immunoeediting. *Nat Immunol* **6**: 722–729
- Feuerer M, Beckhove P, Garbi N, Mahnke Y, Limmer A, Hommel M, Hammerling GJ, Kyewski B, Hamann A, Umansky V, Schirmacher V (2003) Bone marrow as a priming site for T-cell responses to blood-borne antigen. *Nat Med* **9**: 1151–1157
- Hahn B, Trifilo MJ, Zuniga EI, Oldstone MB (2005) Viruses evade the immune system through type I interferon-mediated STAT2-dependent, but STAT1-independent, signaling. *Immunity* **22**: 247–257
- Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K, Akira S (2000) A Toll-like receptor recognizes bacterial DNA. *Nature* **408**: 740–745
- Hoebe K, Du X, Georgel P, Janssen E, Tabeta K, Kim SO, Goode J, Lin P, Mann N, Mudd S, Crozat K, Sovath S, Han J, Beutler B (2003) Identification of Lps2 as a key transducer of MyD88-independent TIR signalling. *Nature* **424**: 743–748
- Hogquist KA, Jameson SC, Heath WR, Howard JL, Bevan MJ, Carbone FR (1994) T cell receptor antagonist peptides induce positive selection. *Cell* **76**: 17–27
- Honda K, Sakaguchi S, Nakajima C, Watanabe A, Yanai H, Matsumoto M, Ohteki T, Kaisho T, Takaoka A, Akira S, Seya T, Taniguchi T (2003) Selective contribution of IFN- α/β signaling to the maturation of dendritic cells induced by double-stranded RNA or viral infection. *Proc Natl Acad Sci USA* **100**: 10872–10877
- Honda K, Takaoka A, Taniguchi T (2006) Type I interferon [corrected] gene induction by the interferon regulatory factor family of transcription factors. *Immunity* **25**: 349–360
- Honda K, Yanai H, Negishi H, Asagiri M, Sato M, Mizutani T, Shimada N, Ohba Y, Takaoka A, Yoshida N, Taniguchi T (2005) IRF-7 is the master regulator of type-I interferon-dependent immune responses. *Nature* **434**: 772–777
- Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, Takeda K, Akira S (1999) Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. *J Immunol* **162**: 3749–3752
- Hotchkiss RS, Coopersmith CM, McDunn JE, Ferguson TA (2009) The sepsis seesaw: tilting toward immunosuppression. *Nat Med* **15**: 496–497
- Hotchkiss RS, Karl IE (2003) The pathophysiology and treatment of sepsis. *N Engl J Med* **348**: 138–150
- Huys L, Van Hauwermeiren F, Dejager L, Dejonckheere E, Lienenklaus S, Weiss S, Leclercq G, Libert C (2009) Type I interferon drives tumor necrosis factor-induced lethal shock. *J Exp Med* **206**: 1873–1882
- Idoyaga J, Suda N, Suda K, Park CG, Steinman RM (2009) Antibody to Langerin/CD207 localizes large numbers of CD8 α ⁺ dendritic cells to the marginal zone of mouse spleen. *Proc Natl Acad Sci USA* **106**: 1524–1529
- Karaghiosoff M, Steinborn R, Kovarik P, Kriegshauser G, Baccarini M, Donabauer B, Reichart U, Kolbe T, Bogdan C, Leanderson T, Levy D, Decker T, Muller M (2003) Central role for type I interferons and Tyk2 in lipopolysaccharide-induced endotoxin shock. *Nat Immunol* **4**: 471–477
- Kawai T, Akira S (2010) The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol* **11**: 373–384
- Kelly-Scumpia KM, Scumpia PO, Delano MJ, Weinstein JS, Cuenca AG, Wynn JL, Moldawer LL (2010) Type I interferon signaling in hematopoietic cells is required for survival in mouse polymicrobial sepsis by regulating CXCL10. *J Exp Med* **207**: 319–326
- Kuhn R, Lohler J, Rennick D, Rajewsky K, Muller W (1993) Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* **75**: 263–274
- Kurts C, Robinson BW, Knolle PA (2010) Cross-priming in health and disease. *Nat Rev Immunol* **10**: 403–414
- Le Bon A, Etchart N, Rossmann C, Ashton M, Hou S, Gewert D, Borrow P, Tough DF (2003) Cross-priming of CD8⁺ T cells stimulated by virus-induced type I interferon. *Nat Immunol* **4**: 1009–1015
- Longhi MP, Trumpfheller C, Idoyaga J, Caskey M, Matos I, Kluger C, Salazar AM, Colonna M, Steinman RM (2009) Dendritic cells require a systemic type I interferon response to mature and induce CD4⁺ Th1 immunity with poly IC as adjuvant. *J Exp Med* **206**: 1589–1602
- Longman RS, Braun D, Pellegrini S, Rice CM, Darnell RB, Albert ML (2007) Dendritic-cell maturation alters intracellular signaling networks, enabling differential effects of IFN- α/β on antigen cross-presentation. *Blood* **109**: 1113–1122
- Mellor AL, Baban B, Chandler PR, Manlapat A, Kahler DJ, Munn DH (2005) Cutting edge: CpG oligonucleotides induce splenic CD19⁺ dendritic cells to acquire potent indoleamine 2,3-dioxygenase-dependent T cell regulatory functions via IFN Type 1 signaling. *J Immunol* **175**: 5601–5605
- O'Garra A, Murphy KM (2009) From IL-10 to IL-12: how pathogens and their products stimulate APCs to induce T(H)1 development. *Nat Immunol* **10**: 929–932
- Randow F, Syrbe U, Meisel C, Krausch D, Zuckermann H, Platzer C, Volk HD (1995) Mechanism of endotoxin desensitization: involvement of interleukin 10 and transforming growth factor β . *J Exp Med* **181**: 1887–1892
- Roger T, Froidevaux C, Le Roy D, Reymond MK, Chanson AL, Mauri D, Burns K, Riederer BM, Akira S, Calandra T (2009) Protection from lethal gram-negative bacterial sepsis by targeting Toll-like receptor 4. *Proc Natl Acad Sci USA* **106**: 2348–2352
- Sato M, Suemori H, Hata N, Asagiri M, Ogasawara K, Nakao K, Nakaya T, Katsuki M, Noguchi S, Tanaka N, Taniguchi T (2000) Distinct and essential roles of transcription factors IRF-3 and IRF-7 in response to viruses for IFN- α/β gene induction. *Immunity* **13**: 539–548
- Schwarz NT, Beer-Stolz D, Simmons RL, Bauer AJ (2002) Pathogenesis of paralytic ileus: intestinal manipulation opens a transient pathway between the intestinal lumen and the leukocytic infiltrate of the jejunal muscularis. *Ann Surg* **235**: 31–40
- Schweichel D, Steitz J, Tormo D, Gaffal E, Ferrer A, Buchs S, Speuser P, Limmer A, Tuting T (2006) Evaluation of DNA vaccination with recombinant adenoviruses using bioluminescence imaging of antigen expression: impact of application routes and delivery with dendritic cells. *J Gene Med* **8**: 1243–1250
- Sims GP, Rowe DC, Rietdijk ST, Herbst R, Coyle AJ (2010) HMGB1 and RAGE in inflammation and cancer. *Annu Rev Immunol* **28**: 367–388
- Steinberg C, Eisenacher K, Gross O, Reindl W, Schmitz F, Ruland J, Krug A (2009) The IFN regulatory factor 7-dependent type I IFN response is not essential for early resistance against murine cytomegalovirus infection. *Eur J Immunol* **39**: 1007–1018
- Takeuchi O, Hoshino K, Kawai T, Sanjo H, Takada H, Ogawa T, Takeda K, Akira S (1999) Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. *Immunity* **11**: 443–451

- Trinchieri G (2007) Interleukin-10 production by effector T cells: Th1 cells show self control. *J Exp Med* **204**: 239–243
- Trinchieri G (2010) Type I interferon: friend or foe? *J Exp Med* **207**: 2053–2063
- van den Broek MF, Muller U, Huang S, Zinkernagel RM, Aguet M (1995) Immune defence in mice lacking type I and/or type II interferon receptors. *Immunol Rev* **148**: 5–18
- Volk HD, Reinke P, Docke WD (2000) Clinical aspects: from systemic inflammation to ‘immunoparalysis’. *Chem Immunol* **74**: 162–177
- Weighardt H, Holzmann B (2007) Role of Toll-like receptor responses for sepsis pathogenesis. *Immunobiology* **212**: 715–722
- Weighardt H, Jusek G, Mages J, Lang R, Hoebe K, Beutler B, Holzmann B (2004) Identification of a TLR4- and TRIF-dependent activation program of dendritic cells. *Eur J Immunol* **34**: 558–564
- Weighardt H, Kaiser-Moore S, Schlautkotter S, Rossmann-Bloek T, Schleicher U, Bogdan C, Holzmann B (2006) Type I IFN modulates host defense and late hyperinflammation in septic peritonitis. *J Immunol* **177**: 5623–5630
- Weighardt H, Kaiser-Moore S, Vabulas RM, Kirschning CJ, Wagner H, Holzmann B (2002) Cutting edge: myeloid differentiation factor 88 deficiency improves resistance against sepsis caused by polymicrobial infection. *J Immunol* **169**: 2823–2827
- Wenzel J, Uerlich M, Worrenkamper E, Freutel S, Bieber T, Tuting T (2005) Scarring skin lesions of discoid lupus erythematosus are characterized by high numbers of skin-homing cytotoxic lymphocytes associated with strong expression of the type I interferon-induced protein MxA. *Br J Dermatol* **153**: 1011–1015
- Wilson NS, Behrens GM, Lundie RJ, Smith CM, Waithman J, Young L, Forehan SP, Mount A, Steptoe RJ, Shortman KD, de Koning-Ward TF, Belz GT, Carbone FR, Crabb BS, Heath WR, Villadangos JA (2006) Systemic activation of dendritic cells by Toll-like receptor ligands or malaria infection impairs cross-presentation and antiviral immunity. *Nat Immunol* **7**: 165–172
- Wingender G, Garbi N, Schumak B, Jungerkes F, Endl E, von Bubnoff D, Steitz J, Striegler J, Moldenhauer G, Tuting T, Heit A, Huster KM, Takikawa O, Akira S, Busch DH, Wagner H, Hammerling GJ, Knolle PA, Limmer A (2006) Systemic application of CpG-rich DNA suppresses adaptive T cell immunity via induction of IDO. *Eur J Immunol* **36**: 12–20
- Yamamoto M, Sato S, Hemmi H, Hoshino K, Kaisho T, Sanjo H, Takeuchi O, Sugiyama M, Okabe M, Takeda K, Akira S (2003) Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science* **301**: 640–643
- Yang H, Hreggvidsdottir HS, Palmblad K, Wang H, Ochani M, Li J, Lu B, Chavan S, Rosas-Ballina M, Al-Abed Y, Akira S, Bierhaus A, Erlandsson-Harris H, Andersson U, Tracey KJ (2010) A critical cysteine is required for HMGB1 binding to Toll-like receptor 4 and activation of macrophage cytokine release. *Proc Natl Acad Sci USA* **107**: 11942–11947
- Zantl N, Uebe A, Neumann B, Wagner H, Siewert JR, Holzmann B, Heidecke CD, Pfeffer K (1998) Essential role of gamma interferon in survival of colon ascendens stent peritonitis, a novel murine model of abdominal sepsis. *Infect Immun* **66**: 2300–2309
- Zuniga EI, Liou LY, Mack L, Mendoza M, Oldstone MB (2008) Persistent virus infection inhibits type I interferon production by plasmacytoid dendritic cells to facilitate opportunistic infections. *Cell Host Microbe* **4**: 374–386