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Enhanced Dendritic Cell Survival Attenuates Lipopolysaccharide-Induced Immunosuppression and Increases Resistance to Lethal Endotoxic Shock

Emmanuel L. Gautier,* Thierry Huby,*† Flora Saint-Charles,* Betty Ouzilleau,† M. John Chapman,*†‡ and Philippe Lesnik2*†‡

Impaired immune function and associated immunosuppression are hallmarks of septic syndromes. As part of an overall deactivation of the immune system, profound depletion of dendritic cells (DCs) occurs in both septic patients and septic mice. Such depletion of DCs is potentially associated with immunosuppression and with failure to induce a protective Th1 immune response; it may equally be predictive of fatal outcome in septic patients. To evaluate the impact of enhanced DC survival on LPS-induced immunosuppression and on survival after LPS-induced septic shock, we created a transgenic mouse model specifically overexpressing the human form of the antiapoptotic protein Bcl-2 in DCs (DC-hBcl-2 mice). DCs derived from DC-hBcl-2 mice exhibited higher resistance to maturation-induced apoptosis after LPS treatment both in vitro and in vivo. Moreover, prolongation of DC survival diminished sublethal LPS-induced DC loss and immunosuppression, with maintenance of the differentiation potential of Th1 cells and enhanced T cell activation. Such modulation of the immune response appears to constitute a key feature of the attenuated mortality observed after LPS-induced shock in DC-hBcl-2 mice. Our study therefore identifies DC death as a key determinant of endotoxin-induced immunosuppression and mortality in mice. The Journal of Immunology, 2008, 180: 6941–6946.

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3 Abbreviations used in this paper: DC, dendritic cell; BMDC, bone marrow-derived dendritic cell; Flt3-L, fms-like tyrosine kinase 3 ligand; PI, propidium iodide; WT, wild type.

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analyzed by flow cytometry. In a second experiment, BMDCs were both serum- and Flt3-L-deprived for 24 to 72 h and apoptosis was analyzed by PI staining and flow cytometry analysis. Mice were injected i.v. with LPS (25 μg/mouse) and apoptosis of DCs was assessed by flow cytometry 18 h later, as described earlier (11). Briefly, splenocytes were recovered and stained with an anti-CD11c Ab and Annexin V FITC to determine the percentage of apoptotic DCs.

**In vivo LPS treatment**

To induce nonlethal systemic inflammation, mice were injected i.v. with LPS (25 μg/mouse). Lethal endotoxic shock was induced in 8-wk-old mice of both genotypes by i.p. injection of LPS (40 mg/kg; *Escherichia coli*). Survival was monitored for 5 wk, and control mice with an anti-hBcl-2 Ab and either a DC marker (CD11c), a B cell marker (CD19), a T cell marker (CD3), or a macrophage marker (CD11b) were stained and assessed by flow cytometry. Such staining revealed specific expression of hBcl-2 in spleen T cells, B cells, macrophages, and DCs from WT and DC-hBcl-2 mice. The shaded profile represents WT mice and the open profile DC-hBcl-2 mice.

**Statistical analysis**

Values are expressed as means ± SEM. The statistical significance of the differences between groups was evaluated using two-tailed Student’s *t* test for unpaired comparisons. Survival data were analyzed using the Kaplan-Meier test. *p* < 0.05 was considered significant.

**Results**

**Generation and characterization of transgenic mice**

Transgenic mice were generated by injection of a construct containing the human Bcl-2 cDNA under the DC-specific murine CD11c promoter into pronuclei of C57BL/6 oocytes (Fig. 1A). Of the three founders obtained, one transmitted the transgene to its progeny, which were termed *DC-hBcl-2* mice. To evaluate the expression of hBcl-2, we performed real-time quantitative PCR analysis on organs containing DCs (spleen, lung, and thymus) and on control tissues known to be devoid of DCs under normal conditions (heart and muscle) from both *DC-hBcl-2* and control mice. Significant expression of hBcl-2 was observed in spleen, lung, and thymus of *DC-hBcl-2* mice only, whereas hBcl-2 cDNA was undetectable in both heart and muscle of *DC-hBcl-2* and control mice (Fig. 1B). To demonstrate protein expression of hBcl-2, we prepared BMDCs from *DC-hBcl-2* and control mice and assessed the presence of hBcl-2 by intracellular staining and flow cytometry. Such staining revealed specific expression of hBcl-2 in BMDCs derived from *DC-hBcl-2* mice (data not shown).

**Increased survival of DCs from DC-hBcl-2 mice**

As Bcl-2 is a key regulator of the mitochondrial death pathway, we hypothesized that *DC-hBcl-2*-derived BMDCs might exhibit higher survival capacity and resistance to apoptosis than those derived from their wild-type (WT) controls. First, to specifically challenge the mitochondrial apoptotic pathway, BMDCs derived from both *DC-hBcl-2* and WT mice were serum- and GM-CSF-deprived for 24, 48,
and 72 h. Annexin V/PI staining revealed ~2-fold, 4-fold, and 3-fold increase in the proportion of viable cells in transgenic BMDCs as compared with controls after 24, 48, and 72 h, respectively (Fig. 2A). As Bcl-2 has been proposed to confer resistance against apoptosis induced by DC activators such as LPS (8) or LPS + IFN-γ (13), BMDCs were incubated with LPS or LPS + IFN-γ and survival was analyzed 96 h after activation. After LPS and LPS + IFN-γ stimulation, BMDCs derived from DC-hBcl-2 mice exhibited enhanced survival (90% and 40%, respectively) as compared with control BMDCs (Fig. 2B). To validate these results in vivo, we injected mice with LPS and measured apoptosis of DCs 18 h later as previously described (11). As shown in Fig. 2C, DCs from DC-hBcl-2 mice were 40% more resistant to LPS-induced apoptosis than were DCs from control mice.

DCs accumulate in DC-hBcl-2 mice and enhance T cell activation
As we demonstrated that BMDCs derived from DC-hBcl-2 mice display increased survival in vitro, we evaluated the potential impact of enhanced DC survival on DC homeostasis in vivo. Flow cytometry analysis of spleen DCs revealed their significant expansion (Fig. 3A, 1.6-fold increase, \( p < 0.05 \)) in DC-hBcl-2 mice as compared with WT controls. In light of the role of DCs as T cell response inducers, we analyzed T cell activation in both DC-hBcl-2 and WT mice. A marked increase in the activation marker CD69 was found among CD3 T cells (\( p < 0.01 \)) and CD4 T cells (\( p < 0.001 \)) in DC-hBcl-2 mice as compared with nontransgenic controls (Fig. 3, B and C, respectively).

**Attenuated DC loss in DC-hBcl-2 mice after nonlethal LPS injection enhanced T and B cell activation**
Injection of a nonlethal dose of LPS has been previously associated with induction of DC apoptosis (11, 14) and subsequent loss of DCs 48 h after injection (6, 14, 15). Such treatment mimics DC loss and immunosuppression observed in severe septic shock. In this setting, DC-hBcl-2 mice and their WT controls were injected i.v. with 25 μg LPS (1.25 μg/g), and the impact on spleen DC content and T cell activation was evaluated 48 h later. Two days after nonlethal LPS injection, we observed a 2.8-fold increase in the DC population in DC-hBcl-2 mice as compared with controls (Fig. 4A, \( p < 0.01 \)). Such a marked increase in the DC population in DC-hBcl-2 mice vs WT mice is greater than that observed in the basal state (Fig. 3A, 1.6-fold increase, \( p < 0.05 \)). As compared with the basal state, DC loss after nonlethal LPS injection in WT and DC-hBcl-2 mice was 65% and 35%, respectively (Fig. 4A). Under these conditions, such attenuated loss of DC in DC-hBcl-2 mice was associated with a greater proportion of activated CD3 T cells (Fig. 4B, \( p < 0.005 \)) and CD19 B cells (Fig. 4C, \( p < 0.05 \)). Independently of mouse genotype, DC content was highly correlated with the proportion of activated T cells (Fig. 4D, \( r = 0.85, p < 0.005 \)) and moderately associated with B cell activation (data not shown, \( r = 0.37, p < 0.05 \)). Moreover, consistent with these results, we observed that splenocytes from DC-hBcl-2 mice exhibited a higher proliferation rate as compared with controls 48 h after in vitro restimulation of splenocyte with LPS (data not shown).

**Attenuated DC loss in DC-hBcl-2 mice is without effect on the systemic cytokine profile after nonlethal LPS injection**
DCs possess the capacity to produce a wide range of immunomodulatory cytokines and to drive lymphocyte cytokine expression. The impact of the attenuated loss of DCs following LPS treatment on systemic cytokine profile was therefore assessed in DC-hBcl-2 and WT mice 48 h after injection. As shown in Table...
DENDRITIC CELLS PROTECT AGAINST ENDOTOXIC SHOCK

FIGURE 4. Attenuated DC loss and enhanced T and B cell activation in DC-hBcl-2 mice after nonlethal LPS injection. A, DC (CD45+CD11c+) content in spleen from WT and DC-hBcl-2 mice after nonlethal LPS injection was determined by flow cytometry. The percentage of DC loss after nonlethal LPS injection as compared with basal state was reported for WT and DC-hBcl-2 mice. B, Activated CD69+ T cells in the spleens of WT and DC-hBcl-2 mice after nonlethal LPS injection. C, Activated CD86+ B cells in the spleens of WT and DC-hBcl-2 mice after nonlethal LPS injection. D, Correlation between DC content and activated T cells in spleens of WT and DC-hBcl-2 mice. * and ** indicate statistically significant differences between the two groups: p < 0.05 and p < 0.01, respectively.

FIGURE 5. Attenuated DC loss and Th1 polarization in the spleens of DC-hBcl-2 mice after nonlethal LPS injection. A, mRNA expression of the DC marker CD11c was evaluated by quantitative PCR in spleens from WT and DC-hBcl-2 mice after nonlethal LPS injection. B, mRNA expression of the transcription factors GATA-3 and TIM-3, which are known to promote Th2 and Th1 development, respectively, by quantitative PCR in spleen from WT and DC-hBcl-2 mice after nonlethal LPS injection. C, Cytokine secretion after splenocyte restimulation by LPS was measured in 48-h supernatants. * and ** indicate statistically significant differences between the two groups: p < 0.05 and p < 0.001, respectively.

Attenuated DC loss and Th1 polarization in the spleens of DC-hBcl-2 mice after nonlethal LPS injection

We next determined the impact of the attenuated loss of DCs observed in DC-hBcl-2 mice on immune polarization and cytokine mRNA expression. First, we confirmed that DC content was greater in the spleen of DC-hBcl-2 mice, as the mRNA coding for the DC-specific marker CD11c was elevated in splenic tissue as compared with that in WT controls (Fig. 5A, p < 0.05). We next analyzed the expression of two transcription factors, GATA-3 and TIM-3, which are known to promote Th2 and Th1 development, respectively. mRNA quantification using quantitative PCR revealed no difference in GATA-3 expression (Fig. 5B), whereas TIM-3 mRNA levels were increased by 60% in spleens of DC-hBcl-2 mice as compared with controls (Fig. 5B, p < 0.001). Such Th1 polarization in DC-hBcl-2 mice is consistent with significant increase in mRNA levels coding for the costimulatory molecule CD86 and the immunostimulatory cytokines IL-12p40, IL-15, and IL-23 as compared with controls (Table II). Additionally, a trend toward increased IL-18 mRNA expression in transgenic mice was observed (Table II). Consistent with enhanced T cell activation and elevated levels of mRNA coding for immunostimulatory cytokine in DC-hBcl-2 mice, we observed enhanced IFN-γ mRNA expression in these mice as compared with WT controls (+75%, Table II). To corroborate these data, splenocytes were recovered from both DC-hBcl-2 and control mice 48 h after nonlethal LPS injection, restimulated in vitro with LPS, and cell supernatants were analyzed for cytokine production. As compared with controls, production of the Th1 cytokines IL-12p40 and IFN-γ was increased, whereas the secretion of the Th2 cytokine IL-4 was decreased in DC-hBcl-2 splenocyte supernatants (Fig. 5C). These data were consistent with the gene expression profile obtained in spleen, and they are in accordance with a Th1 bias and decreased immunosupression after nonlethal LPS injection.

DC-hBcl-2 mice display higher resistance to lethal endotoxic shock

LPS injection is an established surrogate for Gram-negative bacteria-induced septic shock. In this setting, we injected lethal doses of LPS (i.e., 40 mg/kg) to induce septic shock in both DC-hBcl-2 and

<table>
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<tr>
<th>mRNA</th>
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<td>CD86</td>
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<td>IL-12p40</td>
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<td>61 ± 12</td>
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<td>115 ± 14</td>
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<tr>
<td>IL-23p19</td>
<td>108 ± 32</td>
<td>335 ± 150</td>
<td>&lt;0.05</td>
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<td>IFN-γ</td>
<td>79 ± 18</td>
<td>138 ± 18</td>
<td>&lt;0.01</td>
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* AU indicates arbitrary units.
and WT mice and subsequently monitored their survival for 96 h. Transgenic mice overexpressing hBcl-2 in DCs exhibited a marked improvement in survival after LPS-induced septic shock as compared with their WT controls. After 4 days, survival rate was 58% and 7% for DC-hBcl-2 and WT mice, respectively (Fig. 6, p = 0.02).

Discussion

At present, the treatment of septic shock is not optimal, notably due to incomplete knowledge of the dysregulation of the immune system that leads to organ injury and subsequent mortality. The role of DCs in septic shock has recently been highlighted by the fact that these cells are profoundly depleted in both septic mice (3–5) and patients (1, 2). Herein, we provide evidence that enhanced DC survival diminished nonlethal LPS-induced DC loss and immunosuppression, with maintenance of Th1 differentiation potential and enhanced T cell activation, thereby accounting for the decreased mortality observed after LPS-induced septic shock.

It is now well documented that extensive apoptosis of leukocytes, including T lymphocytes and DCs, occurs upon sepsis and endotoxic shock, and thus it may contribute to the immune suppression characteristic of these disorders (16). Indeed, modulation of DC survival in mice has been shown to influence their immunogenicity (7, 8, 17, 18). Herein, we revealed that under basal conditions, mice overexpressing hBcl-2 in DCs (DC-hBcl-2 mice) presented an accumulation of DCs in their spleens in association with increased T cell activation. Moreover, after nonlethal LPS shock, Bcl-2 regulated DC survival after maturation and led both to accumulation of DCs and to enhanced T cell survival.

These observations are consistent with the established function of DCs in regulating both T and B cell responses. The elevated DC content in DC-hBcl-2 mice after nonlethal LPS shock was associated with a Th1 polarization as assessed by increased TIM-3 expression in the spleen, with no change in levels of GATA-3 mRNA. Th1 responses are characterized by IFN-γ secretion, a cytokine primarily produced by activated T cells, and that is associated with enhanced resistance to lethal septic shock in mice (19–21). In this setting, septic shock-associated DC depletion may lead to decreased T cell activation and IFN-γ production, and subsequently to increased mortality. Consistent with this hypothesis, we observed that DC-hBcl-2 mice exhibited higher IFN-γ expression and production than did their controls after nonlethal LPS challenge, thereby suggesting that these mice are more immunocompetent as compared with control mice. Moreover, induction of tolerance in mice by nonlethal LPS injection was associated with immunosuppression characterized by suppressed IFN-γ secretion (22). In this latter study, induction of tolerance by LPS may arise from DC loss and suppression of IL-12 production, as observed in a similar model by Wysocka and colleagues (23). The role of IL-12 expression in DCs is well established and is known to drive Th1 cell differentiation and activation. With respect to this central role for IL-12, immunosuppression after LPS challenge or other types of infection has been shown to be essentially due to decreased IL-12p40 production by DCs (20, 23, 24). In this context, increased IL-12p40 expression and production in the spleens of DC-hBcl-2 mice provide a rationale for decreased immunosuppression after LPS challenge in our transgenic mouse model. This hypothesis is further corroborated by the finding that mRNA levels coding for the immunostimulatory cytokines IL-15 and IL-23p19 were elevated in the spleens of DC-hBcl-2 mice as compared with controls. Moreover, we observed that the production of the prototypic Th2 cytokine IL-4 by DC-hBcl-2 splenocytes was decreased following restimulation in vitro as compared with controls. Considered together, DC-hBcl-2 mice exhibited decreased LPS-associated DC loss, which led to increased mRNA levels of the immunostimulatory cytokines IL-12p40, IL-15, IL-23p19, and IFN-γ; in turn, these cytokines enhanced T and B cell activation. Our results are concordant with recent findings highlighting the fact that IL-12p40-deficient mice show reduced survival rates after septic shock, primarily due to defective IFN-γ production (20). Moreover, IL-15 is necessary for mature DC survival in mice (13). Consequently, IL-15-deficient mice are characterized by decreased DC numbers in blood and spleen, and transgenic overexpression of IL-15 in IL-15-deficient mice restores DC numbers (13). Under these conditions, IL-15 overexpression in mice is associated with enhanced survival following E. coli-induced septic shock (25). In this context, Flt3-L treatment, which enhances differentiation and mobilization of DCs, has been shown to suppress endotoxin-induced immunosuppression (26).

Consistent with the decreased immunosuppression observed in our transgenic model following nonlethal LPS injection, we demonstrated that DC-hBcl-2 mice exhibited marked improvement in survival after LPS-induced septic shock as compared with their WT controls. It is therefore relevant that depletion of DCs has recently been associated with increased mortality following septic shock (27); however, the mechanisms underlying the protective potential of DCs have not been addressed. Consistent with findings in the present study, Scumpia and colleagues found no differences in plasma cytokine concentrations (27). DCs may not dramatically impact the circulating cytokine pool; in contrast, however, maintenance of DC numbers together with their immunostimulatory properties maintains Th1 potential differentiation, IFN-γ production, and confers protection against septic shock. As maintenance of the potential of the immune system by enhancing Th1 lymphocyte survival (19, 28, 29) appears to attenuate septic shock-associated immunosuppression and mortality in mice, we hypothesize therefore that such benefit may be partly mediated by DCs, as they may regulate T cell survival (30–33), and notably through enhanced MHC class II/TCR interactions (30, 34, 35).

Additionally, as shown by the study of Hotchkiss et al. (36), adoptive transfer of apoptotic cells, which exerts antiinflammatory/immunosuppressive effects, has been shown to decrease survival in sepsis. In this context, we can hypothesize that hBcl-2 expression in DCs may protect mice from overt immunosuppression by reduction in the production of apoptotic DCs. Hotchkiss et al. equally demonstrated that the diminished survival of mice associated with adoptive transfer of apoptotic cells was associated with a decreased production of IFN-γ and a slight increase in IL-4 levels, thereby indicating a shift toward a Th2 response (36). Our DC-hBcl-2 mice model mirrors this phenomenon with maintenance of a Th1 propensity that is associated with increased survival; indeed, these findings are consistent with the studies of Bomhhardt

FIGURE 6. Overexpression of hBcl-2 in DCs improves survival after LPS-induced endotoxic shock. Transgenic mice overexpressing the anti-apoptotic protein hBcl-2, under the DC-specific promoter CD11c, underwent LPS-induced septic shock and their survival was monitored for 96 h. Mice overexpressing hBcl-2 displayed a statistically significant improvement in survival as compared with WT controls, p = 0.02.
et al. (19) in a transgenic model of Akt overexpression in T cells, and equally with the work of Moreno et al. (20) in which IL-12 and IFN-γ−/− mice showed higher susceptibility to sepsis. We cannot however exclude the possibility that DCs from DC-hBcl2 mice may have exhibited tolerogenic properties, as splenic IL-10 expression and splenocyte production of IL-10 is increased in DC-hBcl2 mice as compared with controls (data not shown). In this way, DC-hBcl2 mice may have afforded protection against endotoxin-induced cell death via IL-10, as demonstrated by the studies of Oberholzer at al. and of Fujita et al. (37, 38). Additionally, elevated numbers of DCs in all tissues can provide a source both of endotoxin-binding and of endotoxin-degrading proteins such as CD14, MD-2-TLR4-complex, and the LPS-detoxifying enzyme acylacyl hydrolase, respectively (39).

In conclusion, we provide evidence that enhancement of DC survival is associated with decreased immunosuppression following nonlethal LPS injection as assessed by increased immunostimulatory cytokine expression and enhanced T and B cell activation. In a model of LPS-induced septic shock, these findings are associated with increased survival. Our present study therefore identifies the DC as a key player in endotoxin-induced immunosuppression and mortality in mice.

Disclosures

The authors have no financial conflicts of interest.

References


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