Flt3 Ligand–treated Neonatal Mice Have Increased Innate Immunity Against Intracellular Pathogens and Efficiently Control Virus Infections

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Abstract

Flt-3 ligand (FL), a hematopoetic growth factor, increases the number of dendritic cells (DCs), B cells, and natural killer cells in adult mice but the effect in neonates was unknown. We show that FL treatment of newborn mice induced a >100-fold increase in the innate resistance against infection with herpes simplex virus type 1 and *Listeria monocytogenes*. This resistance required interferon (IFN)-α/β for viral and interleukin (IL)-12 for bacterial infections. Long-term survival after viral but not bacterial infection was increased ~100-fold by FL treatment. After treatment, $CD11c^+$/major histocompatibility complex type II^+ and $CD11c^+/B220^+$ DC lineage cells were the only cell populations increased in the spleen, liver, peritoneum, and skin. DC induction was independent of IFNs, IL-2, -4, -7, -9, -15, and mature T and B cells. The data suggest that FL increases the number of DCs in neonates and possibly in other immune-compromised individuals, which in turn improves IFN-α/β– and IL-12–associated immune responses.

Key words: neonatal immune response • DC • IL • virus • bacteria

Introduction

Neonates have an immature immune system. Their IFN production is inefficient in controlling virus replication (1). The number of T and B cells in the secondary lymphoid organs are 1,000–10,000 times lower than in adults and appear too limited in number to mount protective immune responses, where the number and defense potential of specific lymphocytes is crucial (2–6). Hence, neonates are highly susceptible to infection with intracellular pathogens. In newborn mice, B cells are difficult to activate but T cells can be primed at a neonatal or even prenatal stage (7). However, T cell activation requires adequate numbers of dendritic cells (DCs)* or else tolerance is induced (5, 8–10).

Flt3 ligand (FL) is a growth factor that binds to early haematopoietic precursor cells in fetal liver or bone marrow (11, 12). Treatment of adults with FL results in an increase of the relative and absolute number of biologically active DCs, B cells, and NK cells in peripheral tissues (13–15). The adult animals used in these studies had a mature hormone and immune system and cell homing was at equilibrium. It was not clear whether exogenous FL could increase the number of such cell populations in neonatal mice with a developing immune system and whether these cells would be biologically active.

To address these questions, neonatal mice were treated for 7 d with FL and tested for susceptibility to infection with intracellular pathogens. After FL treatment, the nature and quality of resistance was analyzed for short-term innate effects and for survival of the animals. We found that the innate immune system of FL–treated animals was at least 100 times more resistant than controls to infection with HSV-1 or *Listeria monocytogenes*. In these neonatal mice DC lineage cells were the only cell populations increased in the organs tested. The data suggest that FL induces IFN-α/β– and IL-12–associated immune responses in neonates by expanding cells of the DC lineage.

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**Abbreviations used in this paper:* DC, dendritic cell; FL, Flt3 ligand.

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Materials and Methods

Animals. Wild-type, C57BL/6, BALB/c, 129Sv/Ev, and congenic mice with gene-targeted disruptions of the IFN receptor type I (A129), the IFN receptor types I and II (AG129), the RAG-1 ($RAG^{-/-}$), the IFN receptor type I and RAG (AR129), the IFN receptor type II and RAG (GR129), the IFN receptor types I and II, as well as RAG (AGR129) and RAG mice with an additional deletion of the common γc chain ($RAG^{-/-}$ $\gamma c^{-/-}$) were used (16–18). Mice were bred and maintained under specific pathogen-free conditions in the Labortierkunde or the Nagerzentrum, University of Zurich.

Treatment of Newborn Mice with FL. Newborn mice were injected subcutaneously within 24 h of birth and consecutively for 6 d with 1 μg human FL (Immunex) in 50 μl LPS-free PBS.

Animal Challenge Experiments with HSV-1 and L. monocytogenes. HSV-1 F strain was originally obtained from B. Roizman (University of Chicago, Chicago, IL) and propagated on Vero cells (19). For all experiments, purified virus particles were used. Purification of infectious particles was performed by ultra centrifugation on a sucrose density gradient and the virus titer was determined as previously described (20). *L. monocytogenes* obtained from R.M. Zinkernagel (University of Zurich, Zurich, Switzerland) were propagated and titrated using established bacteriological procedures.

Mice were challenged subcutaneously with HSV-1 or intraperitoneally with *L. monocytogenes* at 7 d of age. Surviving animals were scored 21 d after infection with various doses of HSV-1. Bacterial titer in the spleen was determined 5 d after infection with *L. monocytogenes*. The infective dose of *L. monocytogenes* used was 50 CFU per animal, which is 20-fold below the lethal dose required to kill 50% of the 7-d-old C57BL/6 mice (LD_{50}) within 5 d. 10–50 CFU *L. monocytogenes* were used for challenge experiments to evaluate the long-term survival of the 7-d-old C57BL/6 mice.

To determine the biological significance of IL-12 p75, 10 μg monoclonal antibody (10F6) specific to mIL-12 p40 and able to neutralize bioactive IL-12 p75 was administered to mice 1 d before and 1 and 3 d after the infection experiment (21). Recombinant purified human interferon α B/D hybrid (rHuIFN-α B/D; reference 22) was obtained from M.A. Horisberger (Novartis, Basel, Switzerland).

Immunofluorescent Staining and Flow Cytometry. Mice were killed by an overdose of anesthesia. Spleen and liver cells were prepared as previously described (20). In some cases DCs were enriched by depletion and a density centrifugation procedure as previously described (23), except that antibody specific to B220 (see below) was omitted from the depletion mix to ensure that plasmacytoid DCs were present and antibody to CD19 was added instead. To prepare peritoneal lavage cells, the ventral skin was removed and 1 ml PBS in a 1-ml syringe was slowly injected in the abdomen with a 22-G (0.7-mm) needle and thereafter reaspirated. This procedure was repeated twice. A minimum of 3 mice was required for 10 analyses.

Cell analysis was performed using a FACSCalibur® (Becton Dickinson) and the data were analyzed as previously described (23, 24). The following monoclonal antibodies were used: FITC-conjugated hamster IgG group 1, IgG isotype standard anti-trinitrophenol (Cat. No. 11124), FITC-conjugated hamster anti–mouse CD11c (Cat. No. 09704), R-PE–conjugated rat anti–mouse CD86 (B7-2; Cat. No. 553692), PE-conjugated rat anti–mouse I-A/I-E (Cat. No. 06355), FITC-conjugated rat anti–mouse CD19 (Cat. No. 553785), FITC-conjugated rat anti–mouse CD11b (Cat. No. 557396), PE-conjugated rat anti–mouse CD45R/B220 (Cat. No. 553089), FITC-conjugated rat anti–mouse NK-1.1 (Cat. No. 553164), PE-conjugated rat anti–mouse CD3 (Cat. No. 28005B), and R-PE–conjugated rat anti–mouse pan NK cells DX5 (Cat. No. 553858; all from Becton Dickinson and BD Biosciences). Staining was performed according to standard protocols.

Statistics. Data were compared with a two-site *t* test. P-values were indicated as highly significant, <0.0001 (****) and <0.001 (***) and significant, <0.01 (**) and <0.05 (*). Data are given as mean and ± standard deviation where appropriate.

Immunohistochemistry. Freshly removed organs were immersed in Hank's balanced salt solution and snap frozen in liquid nitrogen. For the staining of cell differentiation markers, frozen tissue sections of 5-μm thickness were cut in a cryostat, placed on siliconized glass slides, air dried, fixed in acetone for 10 min, and stored at −70°C. Rehydrated tissue sections were incubated with primary rat monoclonal antibodies against CD45R/B220 (RA3-6B2; BD Biosciences) or anti–mouse CD11c (BD Biosciences) and with primary monoclonal hamster antibodies (N418; reference 2). Primary antibodies were revealed by sequential incubation with goat antibodies against species-specific Igs followed by alkaline phosphatase–labeled donkey antibodies against goat immunoglobulins (Jackson ImmunoResearch Laboratories). Dilutions of anti-Ig reagents were made in Tris-buffered saline containing 5% normal mouse serum. Alkaline phosphatase was visualized using naphthol AS-BI (6-bromo-2-hydroxy-3-naphtholic acid-2-methoxy anilide) phosphate and new fuchsin as substrate, yielding a red color reaction product. Endogenous alkaline phosphatase was blocked by levamisole. Color reactions were performed at room temperature for 15 min with reagents from Sigma-Aldrich. Sections were counterstained with hemalum and coverslips were mounted with glycerol and gelatin.

Results

Treatment with FL Increases IFN Type I and NK-dependent Innate Resistance Against Infection with HSV-1. Newborn animals are highly susceptible to infection with intracellular pathogens. Because FL treatment of adult animals leads to expansion in the number of DCs, NK cells, and B cells, and thus enhanced resistance to pathogens (25–28), a similar approach was tried in newborn mice. To test for resistance against viral infection, 7-d-old FL–treated and control C57BL/6 or BALB/c mice were challenged with graded doses of HSV-1. The survival of animals was determined 3 wk after viral infection. All FL–treated C57BL/6 mice infected with 10^4 PFU of HSV-1 survived. By contrast, 75% of the control mice died (Fig. 1 a). 70% of the FL–treated mice challenged with 10^5 PFU of HSV-1 survived the viral infection for >21 d whereas all the control mice died (Fig. 1 b). The LD_{50} for HSV-1 of naive, 7-d-old C57BL/6 mice was 10^3 PFU of virus whereas that of adult mice was 5×10^6 PFU of HSV-1 (29). Hence, treatment of newborn C57BL/6 mice with FL increases resistance against HSV-1 infection >100-fold. Furthermore, FL–treated BALB/c mice had a significantly higher resistance against HSV-1 infection when compared with PBS controls (Fig. 1 c). Thus, FL increased resistance of neonates against HSV-1 infection to

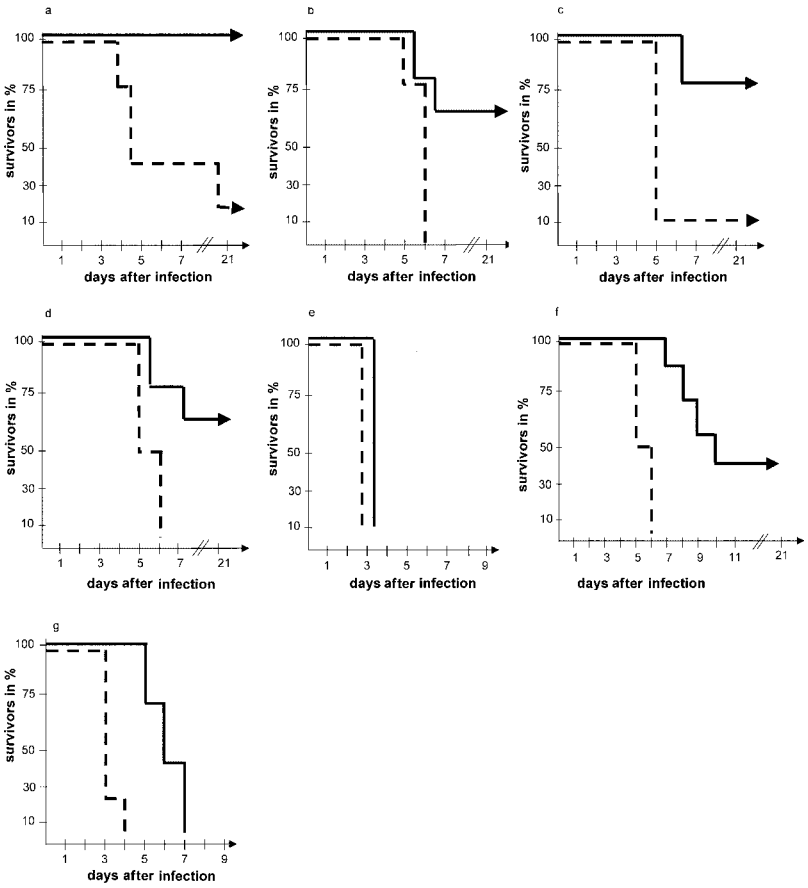

Figure 1. Resistance of FL– or rIFN-α–treated mice against infection with HSV-1. Groups of 6–12 newborn C57BL/6 (a and b), BALB/c (c), C57BL/6 treated with 10^5 IU of rIFN-α at day 7 (d), A129 (e), $RAG1^{-/-}$ (f), or $RAG^{-/-}$ $\gamma c^{-/-}$ (g) were used. Mice in all groups except d were injected daily from day 0 to 6 with FL (solid line) or PBS (broken line) and challenged with 10^4 (a and d), 10^5 (b), 0.5×10^3 (e–g), or 10^2 PFU (c) of HSV-1 at 7 d of age. The survival of mice after viral infection is shown.

adult levels and this biological effect was not restricted to a single mouse strain.

IFN-α/β is crucial for the innate defense against HSV-1 and other viruses (17, 30). To test the effect of IFN-α directly in 7-d-old mice, a single dose of 10^5 IU of the cytokine was administered and the mice were challenged with 10^4 PFU of HSV-1. The majority of rHuIFN-α B/D–treated animals survived the viral challenge whereas all control mice died (Fig. 1 d). In a similar challenge experiment with 10^3 PFU of virus, all rIFN-α–treated animals stayed alive whereas 60% of the control animals died. In contrast, A129 mice with gene-deleted functional type I IFN receptor did not survive any viral challenge regardless of whether the mice were treated with FL or not, and died within a few days (Fig. 1 e). As expected, AG129 mice without a functional IFN system at all were not protected against viral challenge and died within a similar time frame (not depicted). Thus, in the absence of a functional type I IFN system (Fig. 1 e), IFN-γ had no appreciable effect against viral infection in neonates. Mice without functional IFN-γ (G129) were more susceptible to virus infection than wild-type mice (not depicted), indicating cooperative effects between type I and II IFN system in the defense against HSV-1.

Next, the role of specific lymphocytes in the defense against HSV-1 infection was analyzed. RAG-deleted mice lacking mature T and B cells died after challenge with 500 PFU of HSV-1 whereas 30–40% of the FL–treated mice survived (Fig. 1 f). Only 1 out of 10 RAG-deleted mice survived a challenge with 100 PFU of virus whereas 90% of the FL–treated mice remained healthy. RAG-deleted mice with an additional defect on the common γ chain have no significant numbers of NK cells. Untreated $RAG^{-/-}$ $\gamma c^{-/-}$ rapidly succumbed to 500 PFU of HSV-1. FL treatment significantly delayed the onset of the disease but all mice died 2–4 d later (Fig. 1 g). Therefore, the innate resistance against HSV-1 in neonatal mice depends on IFN type I that cooperates with IFN type II, NK cells, or other immune elements associated with the biological function of the (γc) chain.

Increased Innate Resistance of FL–treated Neonatal Mice Against L. monocytogenes Depends on IL-12 and NK Cells. The biological effect of FL treatment on responsiveness to the intracellular pathogen, *L. monocytogenes*, was also examined. The innate immune response against *L. monocytogenes* is well established in adult mice and requires DCs and NK cells. IL-12 produced by DCs licenses NK cells to kill bacteria-infected cells and secrete IFN-γ, which activates macrophages (31, 32). Newborns are very susceptible to infection with *L. monocytogenes*. It was therefore of interest to determine whether FL treatment could increase innate resistance against this pathogen and what immunological parameters might be involved. FL–treated C57BL/6 mice and appropriate controls were infected with 50 CFU of *L.*

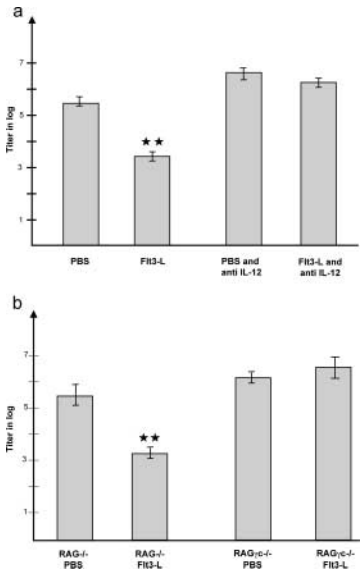

Figure 2. Innate resistance of FL–treated C57BL/6, $RAG^{-/-}$, or $RAG^{-/-}\,\gamma c^{-/-}$ mice against infection with *L. monocytogenes* in the presence or absence of IL-12. Groups of six newborn C57BL/6 (a), $RAG^{-/-}$, or $RAG^{-/-}\,\gamma c^{-/-}$ (b) mice were treated with FL (refer to Fig. 1) or PBS as control and challenged with 50 CFU of *L. monocytogenes* at 7 d of age. Groups of C57BL/6 mice were additionally treated with neutralizing antibodies against IL-12 p70 (anti IL-12). The number of bacteria in spleen was titrated at day 5 after infection.

monocytogenes and the bacterial load present in the spleen was evaluated 5 d later. FL–treated mice had ~100 times fewer bacteria in their spleens than control animals (Fig. 2 a). The increased innate resistance induced by FL was abolished by treating infected mice with neutralizing antibodies specific for IL-12 p40 (Fig. 2 a). In RAG-deleted mice, FL treatment had a similar beneficial effect in the reduction of the bacterial titer as in wild-type mice (Fig. 2 b). In contrast, Flt3- L treatment of mice that lack significant numbers of functional NK cells as well as mature B and T cells ($RAG^{-/-}\,\gamma c^{-/-}$) had no effect on the bacterial titer (Fig. 2 b). Hence, similar to adult animals, the innate immune response against *L. monocytogenes* depends on IL-12 and immune elements associated with a functional (γc) chain in particular significant numbers of NK cells.

FL Increases the Number of $CD11c^+$/MHC-II^+ Cells in Spleen, Liver, Peritoneum, and $CD11c^+$ Cells in Skin. Next, we determined the effect of FL on the cell number in the spleen, liver, peritoneum, and skin from 7-d-old mice. Groups of three to six wild-type C57BL/6 or 129Sv/Ev mice were treated with FL, PBS, or left untreated and individual spleen, liver, and pooled peritoneal lavage cells were analyzed by flow cytometry. Treatment with FL did not influence the total cell number in the spleen or liver when compared with cell numbers in organs from PBS-treated or untreated control animals (not depicted). However, $CD11c^+$ single positive or $CD11c^+$/MHC-II^+ or $CD11c^+$/$CD86^+$ double positive cells were increased three- to fourfold in the spleen and liver (Fig. 3, a–c and Table I). This points to a selective increase in cells of the DC lineage. An increase in the number of the $CD11c^+$ cells was also observed in peritoneum (Table I) and skin (Fig. 4, a and b). In the first few experiments, the spleen or liver from individual animals was analyzed and compared with their pooled cell populations. Statistical analysis of the data showed no difference among the individual or pooled cell populations within the same group of animals. Thus, in subsequent experiments, spleen or liver cells from three to six animals were pooled and analyzed by flow cytometry.

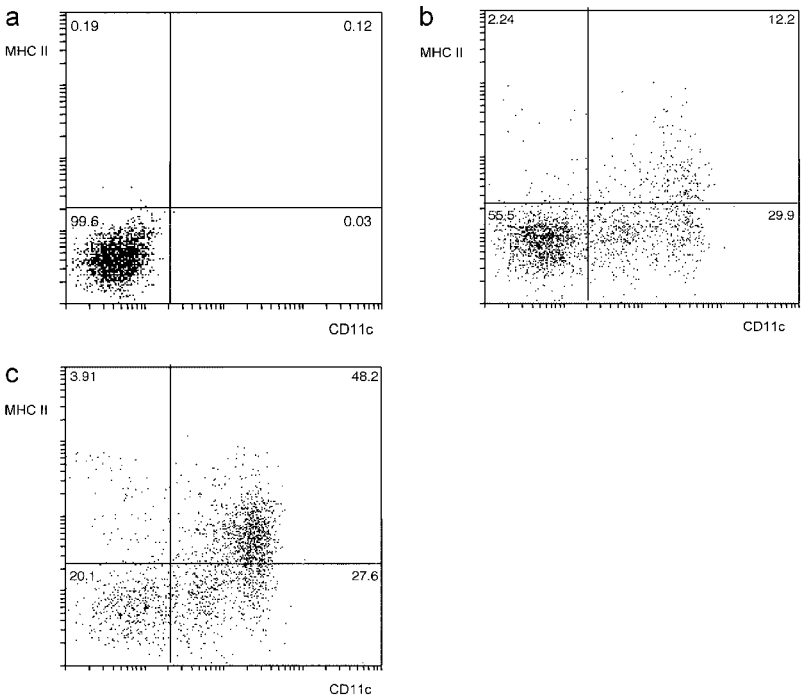

Figure 3. Flow cytometric analysis of $CD11c^+$ and MHC-II^+ spleen cells from 7-d-old C57BL/6 mice. DC-enriched spleen cells from PBS-treated (a and b) or FL–treated (c) mice (refer to Fig.1) were analyzed by flow cytometry unstained (a) or stained with antibody to CD11c and MHC-II (b and c).

Table I. *FL Increases CD11c$^+$/MHC$^+$/CD86$^+$ Cells in Body Compartments*

Cell type	Marker	Percent Flt3[a]	×10^6	Percent BPSC[b]	×10^6	Significance[c]
Spleen						
DC	CD11c	6.9 (0.6)	5.5 (0.5)	1.7 (0.4)	1.4 (0.3)	***
	CD11c/CD86	4.0 (0.1)	3.2 (0.08)	1.3 (0.1)	1.0 (0.1)	***
	CD11c/MHC-II	5.3 (0.2)	4.2 (0.2)	1.4 (0.1)	1.1 (0.1)	***
B	B220	28.9 (2.2)	23.1 (1.8)	26.9 (0.4)	21.5 (0.3)	NS
T	CD3	7.5 (0.5)	6.0 (0.4)	5.5 (1.9)	4.4 (1.5)	NS
NK	NK1.1/DX5	2.4 (0.2)	1.9 (0.2)	2.2 (0.25)	1.8 (0.2)	NS
Peritoneal fluid						
DC	CD11c	17.5 (1.7)		8.9 (2.03)		**
	CD11c/CD86	13.4 (3.6)		7.6 (1.1)		**
	CD11c/MHC-II	14.4 (1.6)		6.5 (2.3)		**
B	B220	ND		ND		
T	CD3	ND		ND		
NK	NK1.1/DX5	ND		ND		
Liver						
DC	CD11c	14.2 (0.9)	10 (0.6)	4.8 (0.3)	3.4 (0.2)	***
	CD11c/CD86	12.3 (0.4)	8.6 (0.3)	4.2 (0.1)	3 (0.7)	***
	CD11c/MHC-II	13.9 (0.3)	9.7 (0.2)	4.7 (0.3)	3.3 (0.2)	***
B	B220	51.6 (1.4)	36.1 (0.9)	51 (0.7)	35.7 (0.5)	NS
T	CD3	8.2 (0.2)	5.7 (0.1)	9.2 (1.3)	6.4 (0.9)	NS
NK	NK1.1/DX5	4.1 (1.7)	2.9 (1.2)	3.6 (0.8)	2.5 (0.6)	NS

Groups of six newborn C57/BL6 mice were injected daily from day 0 to 6 with FL or PBS alone. At 7 d of age, different cell types of individual spleen, pooled peritoneal fluid, or liver were identified by flow cytometry with the cell surface markers indicated. Mean and standard deviations are shown.

[a,b]Proportion (%) of marker-positive cells or absolute cell numbers in organs (×10^6) analyzed from FL– or PBS-treated mice.

[c]Significance(s) as determined in Materials and Methods.

The FL–mediated Increase of CD11c$^+$ Cells Is Independent of Mature B and T Cells, IFN, or IL-2, -4, -7, -9, and -15. In newborn mice, the IFN system and the T and B cell compartment are not fully mature. Therefore, we asked whether the effect of treatment with FL was dependent on mature B and T cells or on a functional IFN system, using genetically modified mice lacking these components. Results in Table II show that neither mature B nor T cells nor

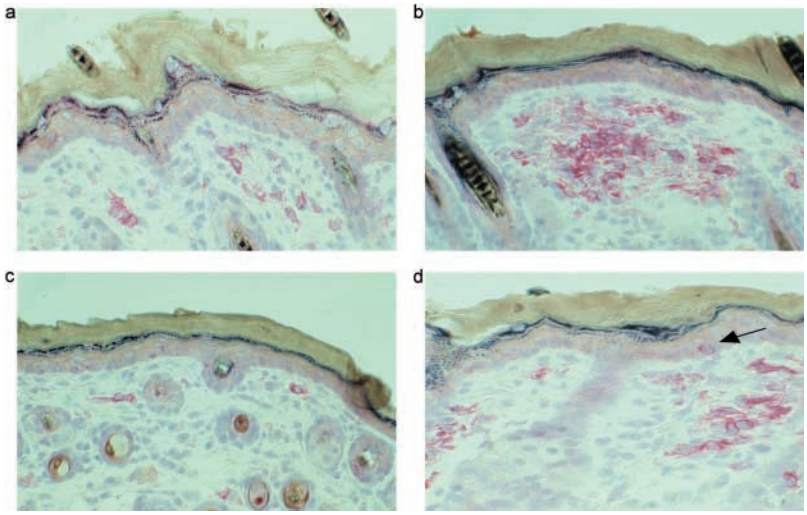

Figure 4. Immunohistology of skin from 7-d-old C57BL/6 or RAG$^{-/-}$ mice treated with antibody to CD11c. Skin sections from C57BL/6 (a and b) or RAG$^{-/-}$ mice (c and d) treated with PBS (a and c) or FL (b and d) were stained with antibody to CD11c, a pan-specific marker for DCs. Note the CD11c$^+$ red cells with dendrites present in dermis as single cells (a and c), clusters (b and d), or occasionally as single cells in epidermis (arrows).

the type I or II IFN system were necessary for FL to increase the level of the $CD11c^+$ cells (Table II and Fig. 4, c and d). In addition, functional IL-2, -4, -7, -9, and -15 ($RAG^{-/-}$ $\gamma c^{-/-}$) were not necessary for this effect. Most $CD11c^+$ cells were double positive for MHC-II (80–90%) or CD86 (70–80%) in the spleen and liver from all mouse strains and all compartments analyzed (Fig. 4 and Table II).

FL Increases the Number of $CD11c^+/B220^+$ Cells in Spleen, Liver, Peritoneum, and $B220^+$ Cells in Skin. Besides $CD11c^+$/MHC-II^+ DCs, treatment of adult mice with FL increases B cells and NK cells. B cells were detected by the antibody B220, a typical "pan" B cell marker in wild-type mice (Fig. 5, a and b). Interestingly, $B220^+$ cells were also found in RAG-deficient mice, which are unable to produce mature B cells (Fig. 5, c and d and Fig. 6, a–d). Additional analysis revealed that some of these cells were also $CD11c^+$, a combination resembling the recently discovered plasmacytoid cells, a major producer of IFN type I and potential precursor of DCs (33). Similar cells were found in mice deleted of the RAG or (γc) receptor (Fig. 6 and not depicted).

The number of NK cells was not affected by the treatment with FL in newborn wild-type mice (Table I). IL-15, an IFN-dependent cytokine is crucial for the development of NK cells. Because newborn mice have an immature IFN system, IL-15 might be very low and therefore may restrict NK cell development. This view was supported by the fact that the number of cells, positive for the NK cell markers, was similar in wild-type mice and animals that are unlikely to make IL-15 (AGR129) or those with a deleted receptor for IL-15 ($RAG^{-/-}$ $\gamma c^{-/-}$; reference 34). Therefore, DCs with the well-known markers $CD11c^+$/MHC-II^+ and cells that resemble plasmacytoid cells ($CD11c^+$/$B220^+$) were found elevated in all organs tested of FL–treated animals (Figs. 3–6). This was in contrast to FL treatment of adult mice that resulted in increases of DCs, B cells, and NK cells.

FL Treatment Increases the Long-Term Survival of Infection with HSV-1 but Not of L. monocytogenes. Flow cytometry analysis indicated that in the neonates analyzed only $CD11c^+$ cells were increased in the spleen, liver, peritoneum, and skin after treatment with FL. This led to a significant increase in the innate resistance against HSV-1 and *L. monocytogenes* (Figs. 1 and 2). Next, we determined the effect of FL treatment on the long-term survival of the infected animals. HSV-1–infected animals that resisted the challenge for 7–10 d survived for >21 d (Fig. 1). Interestingly, >90% of FL–treated RAG mice survived virus doses that were lethal to untreated mice, suggesting that the innate immunity, in particular IFN type I and most probably NK cells, were decisive for survival in neonatal animals. Hence, FL treatment could increase both innate and long-term resistance against virus infections in neonates in the presence or absence of specific immunity.

FL–treated and control mice were challenged with 50 PFU of *L. monocytogenes* and the survival of the animals was

Table II. *FL Increases DCs Independent of IFN, IL-2, -7, -9, -15, and Mature B and T Cells*

Mouse strain[a]	Immunological element[b]				Increase of DC FL/PBS[c]	
	RAG	IFN type I	IFN type II	γc	Spleen[d]	Peritoneal fluid
C57/BL6	+	+	+	+	3.9	3.1
129Sv/Ev	+	+	+	+	3.3	3.3
A129	+	−	+	+	2.6	2.6
AG129	+	−	−	+	2.5	3.2
$RAG^{-/-}$	−	+	+	+	2.1	2.4
$RAG^{-/-}$ $\gamma c^{-/-}$	−	+	+	−	2.6	3.8
AR129	−	−	+	+	3.7	3.6
GR129	−	+	−	+	4.3	2.4
AGR129	−	−	−	+	2.9	1.5

[a]Groups of newborn mice were treated as described in Table 1.
[b]Immunological elements present as wild-type (+) or gene deleted (−) are summarized. Wild-type C57BL/6, 129Sv/Ev, and congenic mice with gene-targeted disruptions of the IFN receptor type I (A129), the IFN receptor types I and II (AG129), the RAG-1 ($RAG^{-/-}$), the IFN receptor type I and RAG (AR129), the IFN receptor type II and RAG (GR129), the IFN receptor types I and II as well as RAG (AGR129), and $RAG^{-/-}$ mice with an additional deletion of the common γc chain ($RAG^{-/-}$ $\gamma c^{-/-}$) were used. At day 7, spleen cells of individual organs or pooled cells collected from peritoneum were analyzed for the presence of $CD11c^+$/MHC-II^+ cells by flow cytometry.
[c]Increase of relative cell numbers of $CD11c^+$/MHC-II^+ cell populations (DC) in spleen and peritoneal fluid of FL– and PBS-treated mice was calculated as in Table 1.
[d]The increase of absolute cell numbers in the spleen was highly significant.

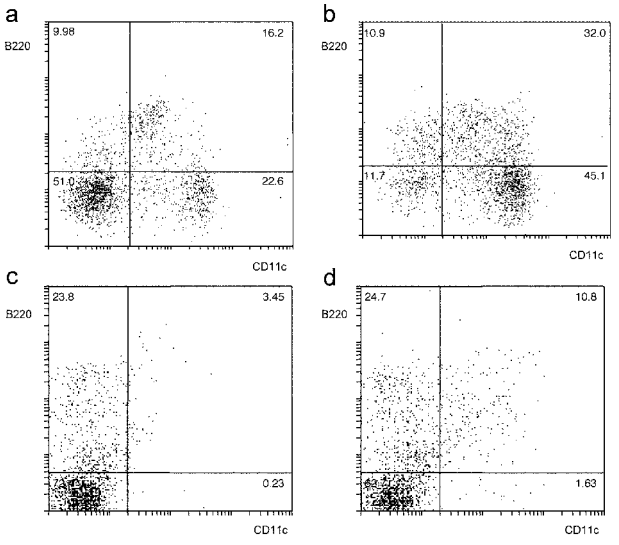

Figure 5. Flow cytometry analysis of $B220^+$ and MHC-II^+ spleen cells from 7-d-old C57BL/6 and $RAG^{-/-}$ mice. DC-enriched spleen cells from C57BL/6 (a and b) or spleen cells from $RAG^{-/-}$ mice (c and d) treated with PBS (a and c) or FL (b and d; refer to Fig. 1) were stained with antibody to CD11c and BB20 and analyzed by flow cytometry.

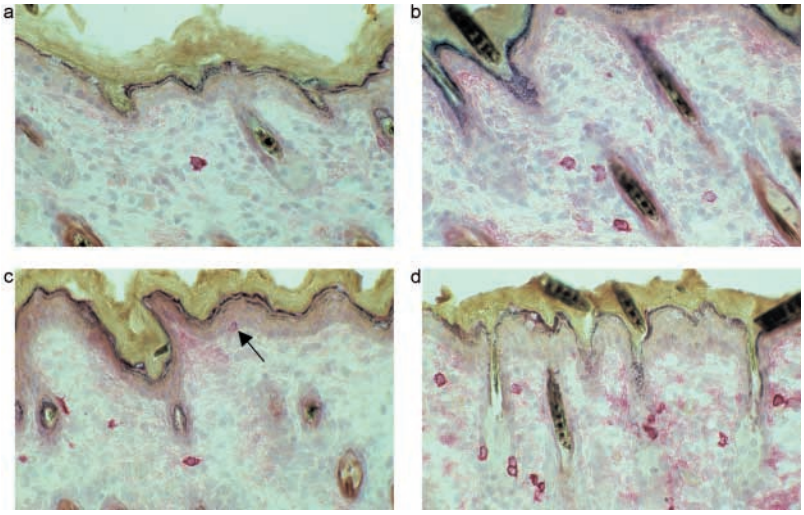

Figure 6. Immunohistology of skin sections from 7-d-old C57BL/6 or $RAG^{-/-}$ mice treated with antibody to B220. Skin sections from C57BL/6 (a and b) or $RAG^{-/-}$ mice (c and d) treated with PBS (a and c) or FL (b and d) were stained with antibody to B220. Note that the $B220^{+}$ red cells with dendrites are present in dermis as single cells (a–d) or occasionally in epidermis (d, arrow) in both mouse strains.

monitored. The control animals rapidly died after bacterial infection whereas the FL–treated animals survived for a longer period of time. But only a few animals survived the challenge (Fig. 7). Challenge experiments with 10–30 PFU of bacteria led to a somewhat expanded life span but no significant long-term survival of animals was observed. Therefore, even though FL treatment resulted in a higher innate resistance against *L. monocytogenes* that led to an increased innate immunity, long-term survival was not achieved as seen in viral infections.

Discussion

In newborns, the biological elements responsible for susceptibility to infection with intracellular pathogens are ill defined. The immature IFN system and the relative low number of immune cells, in particular of DCs (5, 8, 10), were considered important factors limiting resistance against infection with intracellular pathogens. FL, a molecule that induces DCs, B cells, and NK cells in adults (11, 15) was thus tested for its effect in newborn mice.

After FL treatment, the innate resistance against both HSV-1 and *L. monocytogenes* was increased >100-fold. As in adults, the innate immune response of neonates against HSV-1 depends predominantly on type I IFN whereas that against *L. monocytogenes* depends on IL-12. This suggested that FL treatment increased or activated DC lineage cells able to induce IFN-α/β and IL-12.

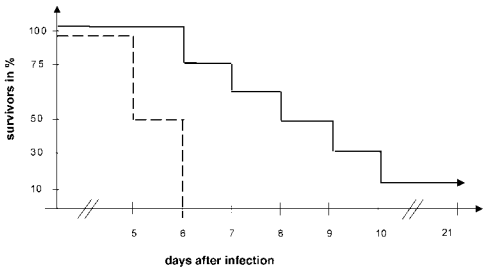

Figure 7. Resistance of FL–treated C57BL/6 mice against infection with *L. monocytogenes*. Groups of six to eight newborn C57BL/6 mice were injected daily from day 0 to 6 with FL (solid line) or PBS (broken line) and challenged with 50 CFU of *L. monocytogenes* at 7 d of age. The survival of mice after infection is shown.

In contrast to adult animals, cell analysis by flow cytometry and immunohistology showed that only DC lineage cells, but not NK cells or B cells, were increased in the spleen, liver, peritoneum, or skin after FL treatment. It is well known that IL-7 is required for the development of T (35) and B cells (36) whereas IL-15 is necessary for the maturation of NK cells (37). Both IL-7 and IL-15 might be regulated by IFN regulatory factors (38). Because of the immature IFN system that induces IFN regulatory factors and the rapid development of T and B cells between birth and day 7 (5), IL-7, IL-15, and possible other factors necessary for the development of B cells and NK cells are limiting. We speculate that the application of external FL to the developing immune system expands additional Flt3-$receptor^{+}$ precursors to further developmental stages (13), but the differentiation to NK cells or B cells may not be possible in neonates due to limiting IL-15 and IL-7.

Hence, after treatment with FL for 7 d, the only differentiated cells that appeared in increased numbers in the periphery were DCs and putative plasmacytoid cells. This suggested that the development of these cells was largely independent of IFN, IL-7, and IL-15. This was verified by injection of FL to animals devoid of a functional IFN system and $RAG^{-/-}$ (AGR129; references 16 and 17), as well as animals devoid of RAG-$1^{-/-}$ and $\gamma c^{-/-}$ (Table II and reference 39). However, it should be noted that the Flt3-$receptor^{+}$ cell population of neonates present in the bone marrow, liver, or spleen may differ from those of adults and their maturation might be differently regulated (40–42). Immunohistology, bone marrow cultures, or treatment of neonatal mice with selected hormones, notably IL-7 and IL-15 together with FL, should help clarify this issue.

The cells stimulated by FL were biologically functional in vivo as shown by infection experiments (Figs. 1 and 2). For the initial control of HSV-1 replication and immune

stimulation, IFN type I is absolutely essential (17, 30). Newborn animals devoid of a functional IFN type I system died very rapidly regardless of treatment with FL and despite the fact that DC lineage cells were up-regulated overall. The role of IFN type I in controlling viral replication was further shown by the enhanced survival of naive wild-type animals treated with rHuIFN-α B/D (Fig. 1). The increased number of FL–induced DCs in RAG1$^{-/-}$ γc$^{-/-}$ might be responsible for the increased production of IFN or induction of this cytokine in other cells and thus for a longer survival time of these animals (Fig. 1 and Table II). For the innate defense of *L. monocytogenes* in adults, and as shown here for neonates, IL-12, possibly derived from DCs (32), is required for NK cell activation and IFN-γ–dependent killing (31).

B220$^+$ plasmacytoid DCs of adults produce high amounts of IFN type I (33) whereas more conventional DCs produce IL-12 (43). Purified populations (23) of splenic DCs from 7-d-old animals stimulated in vitro with virus or CpG also produced high amounts of IFN and IL-12, respectively (unpublished data). Data from flow cytometric analysis and immunohistology implied that CD11c$^+$/B220$^+$ cells present in various organs might be responsible for IFN-α/β production whereas the CD11c$^+$/MHC$^+$ cells could be the source of IL-12 (Figs. 3–6 and Tables I and II). However, data from preliminary FACS® analysis show that more than two DC populations were present in the spleen of 7-d-old mice. Their biological potential awaits analysis in vitro as well as in vivo.

To control intracellular pathogens over extended periods of time, the innate response may need specific immunity to be effective. For survival against lethal infection with HSV-1, a single dose of rHuIFN-α B/D was sufficient for survival of wild-type mice. Interestingly, significant numbers of FL–treated RAG-deficient mice survived the infection for >21 d, indicating that the innate immunity is decisive in the defense of this virus (Fig. 1). This is in contrast to infection with *L. monocytogenes* (Fig. 7). Even though the innate immunity was effective (Fig. 2), long-term survival was not significantly boosted by FL treatment. We speculate that the relatively low number of T cells or their developmental stage in these animals might be one cause (6, 44). SCID mice have been shown to cope with chronic *L. monocytogenes* infection by granuloma formation (45). However, no granuloma formation was detected in our mice after bacterial infection (unpublished data). Therefore, in contrast to virus infection, innate immunity to *L. monocytogenes* may provide some protection but adequate specific immunity is essential for long-term survival. Granuloma formation as a rescue pathway in neonates may have a limited potential (46).

FL has been used in clinical trials (14) and is well tolerated with little if any side effects, even when used at high concentrations. Newborn mice treated daily with 10 μg FL rather than 1 μg were healthy and the number of DCs in various organs was increased four- to fivefold (unpublished data). Therefore, clinical application of FL might be beneficial for two age categories, the newborn and elderly, which are two populations at risk for frequent infection with intracellular pathogens, notably viruses.

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