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IL-2 production by dendritic cells is not critical for the activation of cognate and innate effectors in draining lymph nodes

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Dendritic cells (DC) are unique antigen-presenting cells capable of triggering NK cell effector functions and priming naive T cells *in vivo*. Microbial stimulation induces early IL-2 production by mouse DC. Previous reports demonstrated that IL-2 is enriched at the site of DC/T cell interaction and promotes allogeneic T cell proliferation. However, the direct role of DC-derived IL-2 in the differentiation of cytotoxic T lymphocytes and in NK cell triggering *in vivo* has not been investigated. Lipopolysaccharide (LPS) stimulation of mouse bone morrow-derived DC results in early IL-2 production unless IL-4 is introduced in DC cultures. Here we show that IL-2 produced by LPS-activated DC is dispensable for cognate T cell responses since IL-2 loss of function DC elicit OVA-specific Tc1 effector and memory lymphocytes in draining lymph nodes in a setting where *ex vivo* cultured DC do not transfer antigens to host DC. Moreover, adoptively transferred IL-2 loss of function DC maintain their capacity to trigger NK cell proliferation/recruitment in lymph nodes. Therefore, immediate inducible IL-2 production by DC following microbial infection might play a regulatory role at ports of entry rather than in secondary lymphoid organs.

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Abbreviations: **BM-DC:** bone marrow-derived DC · **CLN:** contralateral lymph node · **CpG ODN:** CpG oligonucleotides · **DLN:** draining lymph node · **IL-2$^{-/-}$ DC:** interleukin-2 loss of function DC · **MCMV:** mouse cytomegalovirus · **Tc1:** type 1 cytotoxic T lymphocytes secreting IFN-γ · **Treg:** regulatory T cells

Introduction

Dendritic cells (DC) play a pivotal part at the interface between innate and cognate immune responses [1]. They undergo a maturation reprogramming in response to microbe-associated molecular patterns such as inflammatory cytokines, microorganisms or microbial products [2, 3]. This maturation process is associated with the DC loss of phagocytic capacities, but with efficiency of antigen processing and presentation in MHC class I and II pathways [4]. It also influence

expression of cytokines and chemokines essential for the regulation of migratory patterns and T cell immune responses [2, 5].

A global gene expression profiling of immature and activated DC at different time points following bacterial encounter enabled F. Granucci *et al.* [6] to find an early upregulation of IL-2 mRNA. Bioactive IL-2 was shown to be secreted by DC *in vitro*, and *in vivo* between 4 and 6 h after stimulation with Gram-negative bacteria but not with inflammatory cytokines. DC-derived IL-2 was critical for the proliferation of allogeneic T cells, and was found to be enriched at the DC/T cell interface during *in vitro* cognate interaction. Previous findings suggested that DC may play a dual role in promoting both antigen-driven and homeostatic cytokine-driven T cell proliferation [7].

The role of DC-derived IL-2 in T cell priming is supported by the fact that mouse cytomegalovirus (MCMV) infection of DC prevents IL-2 production and interferes with DC-mediated T cell activation [2]. However, direct evidence of the paracrine role of IL-2 in the DC-mediated T cell priming *in vivo* has not been brought up to date. Therefore, we addressed the question of the role of early IL-2 production by LPS-activated DC in type 1 cytoxic T cells (Tc1) priming and memory responses in draining LN (DLN). Moreover, since we showed that activated bone marrow-derived DC (BM-DC) can recruit and activate LN NK cells [8], we examined the part played by IL-2 in DC-mediated NK cell activation. Using IL-2 loss of function (IL-$2^{-/-}$) DC, we ruled out a major role of IL-2 in DC-mediated NK cell proliferation/recruitment and peptide-specific, H-2-restricted CTL differentiation in LN.

Results

Bioactive IL-2 is produced by mouse BM-DC in response to TLR4 and 9 ligands

Previous studies reported that IL-2 production is the hallmark of bacterially activated DC [2]. We induced DC activation by triggering various TLR [TLR4 by LPS, TLR5 by flagellin, TLR9 by CpG oligonucleotides (CpG ODN)] or inflammatory cytokine receptors (using TNF-α) that might be transduced during bacterial infections. The binding of all these receptor ligands enhanced the cell surface expression of I-A^b MHC class II molecules (not shown). However, only LPS and CpG ODN could trigger IL-2 secretion from BM-DC (Fig. 1A). The inflammatory cytokine TNF-α, involved in septic shock induced by Gram-negative bacteria, could not promote IL-2 secretion either (Fig. 1A). Nanogramm levels of LPS were sufficient to induce around 500 pg/10^6/mL/24 h of IL-2 production by BM-DC in the extracellular milieu (Fig. 1A, B).

As suggested by Granucci *et al.* [6] studying transcription of IL-2 mRNA in D1 cells at various time points after bacterial encounter, IL-2 production was rapid (within hours) and transient (the first 4 h) (Fig. 1C) after stimulation of BM-DC with LPS. Early IL-2 production by LPS-activated DC was functional and promoted the proliferation of CTLL-2 clones *in vitro* (Fig. 1D). Finally, IL-2 production by mouse BM-DC was not specific for C57BL/6 mouse strain, since BALB/c derived-BM-DC also produced high amounts of IL-2 after activation by LPS (Fig. 1E). Altogether, mouse BM-DC can secrete bioactive IL-2 readily within hours following engagement by TLR4 and 9 ligands *in vitro*.

IL-4 markedly reduced DC responsiveness to LPS for IL-2 production

We have shown *in vitro* and *in vivo* that DC-mediated NK activation occur independently of microbial infection or IL-12 production [9], provided DC are exposed to IL-4 [8, 9]. Moreover, during infections, such as that with *Mycobacterium tuberculosis*, IL-4 is produced by NKT innate effectors and plays a role in modulating DC functions [10]. Therefore, we investigated the capacity of BM-DC propagated in IL-4 to produce IL-2 after stimulation with LPS. As shown in Fig. 2B, the presence of IL-4 or IL-13 during the differentiation or just for a short stimulation prevented BM-DC from responding to LPS for IL-2 production. Similar results were achieved using stimulation with CpG ODN (not shown). It is important to note that BM-DC cultured using recombinant GM-CSF (Fig. 2B) secreted lower amounts of IL-2 than the DC produced with GM-CSF secreted from J558 cells (Fig. 2A), maybe because they displayed a more mature phenotype (50% of cells expressing CD40 molecules) prior to LPS stimulation. Therefore, the paradigmatic Th2 cytokines prevent BM-DC from responding to LPS for IL-2 secretion.

IL-$2^{-/-}$ DC prime naive $CD8^+$ T cells in vivo and induce memory responses

The role of IL-2 produced by early bacteria-stimulated DC in $CD4^+$ and $CD8^+$ T cell activation was demonstrated by analyzing the differential capacity of WT (IL-$2^{+/+}$) versus IL-$2^{-/-}$ DC to stimulate alloreactive T cells in primary MLR [6]. However, the role of early IL-2 production by DC stimulated with LPS in eliciting primary $CD8^+$ T cell responses in DLN has not been adressed. As shown in Fig. 3, BM-DC propagated from IL-$2^{+/+}$ or IL-$2^{-/-}$ mice exhibited a similar phenotype after 13 days in culture, with low expression of I-A^b and costimulatory molecules, and a marked response to LPS. The DC cultures, whether derived from WT or IL-$2^{-/-}$ mice, contained up to 10–18% of contaminating cells,

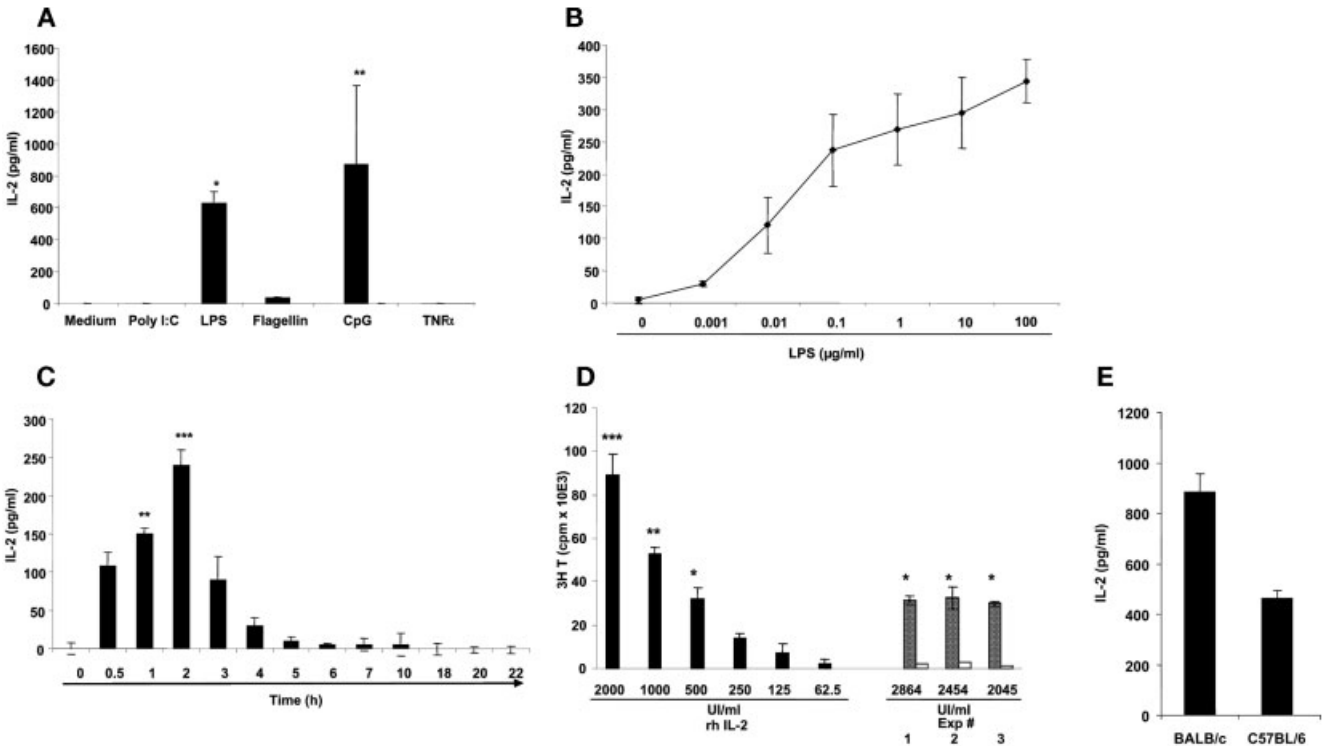

Figure 1. Rapid and transient IL-2 production by mouse BM-DC stimulated with TLR4 and 9 ligands. Mouse BM-DC were propagated from BM precursors in IMDM culture medium and 30% J558-mGM-CSF supernatants from 13-day cultures. BM-DC at a concentration of 4×10^5/mL were activated for 24 h with various stimuli (A), or with increasing dosages of LPS (B). (C) BM-DC were activated with 10 μg/mL of LPS over different time periods. After the activation time, BM-DC were washed three times and replated in LPS-free culture medium for 24 h. (D) The bioactivity of the secreted IL-2 was tested in a CTLL-2 proliferation assay by measuring the incorporation of [^{3}H]thymidine after a 5-day incubation of CTLL-2 with BM-DC culture supernatants containing various dosages of IL-2 (as shown in x-axis). Statistical analyses showed significant differences at $p<0.05$ (*), $p<0.01$ (**) or $p<0.001$ (***) using Kruskal-Wallis tests by comparing CTLL-2 lymphocyte proliferation in the presence or absence of increasing doses of rhuIL-2 (black bars) or supernatants from LPS-stimulated BM-DC containing the indicated amounts of IL-2 in 3 different experiments (gray bars), or supernatants from LPS-free BM-DC cultures (white bars). (E) IL-2 secretion by BM-DC derived from different mouse strains (C57BL/6 and BALB/c) was measured after 24 h of LPS (10 μg/mL) stimulation. Each graph represents pooled data of at least three independent experiments.

which were not T, B or NK cells, *i.e.*, cells producing IL-2 upon LPS stimulation (not shown).

Since the adoptive transfer of DC stimulated for 2 h in LPS were performed in all experiments, we verified that a significant proportion of *ex vivo* propagated DC are indeed reaching the DLN using eGFP-expressing DC derived from FM131 green mice. Prior to footpad inoculation, all CD11c$^+$/I-A^{b+} DC were GFP positive in flow cytometry (Fig. 4A).The cellularity of the DLN increased rapidly upon eGFP-DC inoculation by three-, five- and sixfold at 1, 2 and 3 h post injection, respectively (compared to contralateral LN and baseline, not shown), while the percentages of GFP-expressing cells were $0.01 \pm 0.01\%$, $0.2 \pm 0.01\%$ and $0.04 \pm 0.01\%$, respectively. Half of these GFP$^+$ cells were indeed CD11c$^+$/I-A^{b+} DC (Fig. 4B). Therefore, about 1% of *ex vivo* propagated LPS maturing DC can reach the DLN within 2 h.

The footpad inoculation of 3×10^5 BM-DC activated for 2 h in LPS (^{2hLPS}DC) derived either from IL-2$^{+/+}$ or IL-2$^{-/-}$ mice and pulsed with OVA peptides, promoted a significant expansion of H-2^b-restricted, OVA-specific CTL among CD8$^+$ T cells in the DLN ($1.2 \pm 0.74\%$ or $11\,951 \pm 8424$ cells in IL-2$^{+/+}$ *versus* $0.85 \pm 0.32\%$ or $11\,823 \pm 8900$ cells in IL-2$^{-/-}$ mice, $p>0.05$, Fig. 5) 5 days later. Immature DC did not allow any priming of CD8$^+$ T cells (not shown). Staining with irrelevant soluble fluorescent tetramers did not detect more than 2000 H-2^b-restricted, tyrosinase-related protein 2 (TRP2)-specific CD8$^+$ T cells (not shown). The capacity of LN mononuclear cells to produce IFN-γ in response to OVA peptides was markedly enhanced in homolateral LN, whether or not DC were producing IL-2 (Fig. 6A). PBS, immature DC or unpulsed ^{2hLPS}DC did not promote OVA-specific effector Tc1 lymphocytes (not shown). Moreover, T cell priming achieved using ^{2hLPS}DC was comparable to that obtained with 24-h LPS-stimulated BM-DC (^{24hLPS}DC) (not shown).

To exclude an antigenic transfer between adoptively administered DC and host DC, we injected, at day 0, 10^6

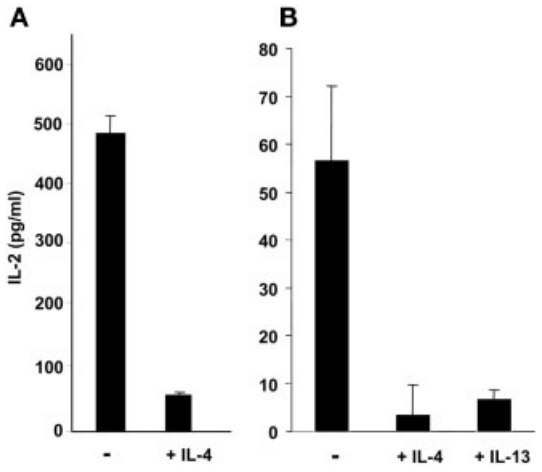

Figure 2. BM-DC propagated in the presence of IL-4 are markedly inhibited for IL-2 production. BM-DC were generated with J558-mGM-CSF for 13 days as described in Fig. 1. IL-4 was added during the last 3 days of culture (A). BM-DC were generated with GM-CSF, GM-CSF + IL-4, or GM-CSF + IL-13 for 6 days (B). BM-DC at a concentration of 4×10^5/mL were then activated with LPS for 24 h. Supernatants were harvested and IL-2 production was assessed using ELISA kits. Each graph represents pooled data of at least three independent experiments.

OT-1 $CD8^+/V\beta5.1^+$ cells into β_2-microglobulin$^{-/-}$ mice. At day 1, 3×10^5 ^{2hLPS}DC and ^{24hLPS}DC propagated from IL-$2^{+/+}$ or IL-$2^{-/-}$ transgenic mice were pulsed or not with OVA peptides and inoculated into the footpad. After 18 h, up to 17–30% of OVA-specific TCR transgenic OT-1 cells of the DLN up-regulated CD69 molecules, regardless of the capacity of DC to produce (^{2hLPS}DC IL-$2^{+/+}$) or not IL-2 (^{2hLPS}DC IL-$2^{-/-}$, ^{24hLPS}DC IL-$2^{+/+}$) (Fig. 6B).

The role of DC-derived IL-2 during the priming phase for the generation of memory $CD8^+$ T cell responses has also been addressed. C57BL/6 mice were immunized s.c. at day 0 with either 3×10^5 ^{2hLPS}DC derived from IL-$2^{+/+}$ or IL-$2^{-/-}$ animals and pulsed or not with OVA peptides. After 5 weeks, animals were rechallenged (in the footpad) with 3×10^5 ^{24hLPS}DC IL-$2^{+/+}$ pulsed with OVA. After 36 h, the levels of IFN-γ secretion *in vitro* by LN cells were OVA peptide dependent and specific, and comparable, regardless of whether the immunizing DC could or could not produce IL-2 (Fig. 6C). We conclude that early IL-2 production by DC during the priming phase of $CD8^+$ T cells is not mandatory to promote memory responses to synthetic peptides. Altogether, BM-DC, after a 2-h stimulation in LPS, are able to trigger the differentiation of effector Tc1 $CD8^+$ cells and memory T cell responses, but independently of their IL-2 production.

IL-$2^{-/-}$ DC promote NK cell triggering in LN

We have reported that IL-4-propagated BM-DC can promote recruitment and/or proliferation of LN-resident NK cells, and that this NK cell activation was enhanced when BM-DC were maturing in LPS [8]. In this setting, LPS did not directly activate LN NK cells. Therefore, we addressed the role of IL-2 production by LPS-stimulated BM-DC in LN NK cell triggering. The footpad inoculation of 3×10^5 ^{2hLPS}DC IL-$2^{-/-}$ did

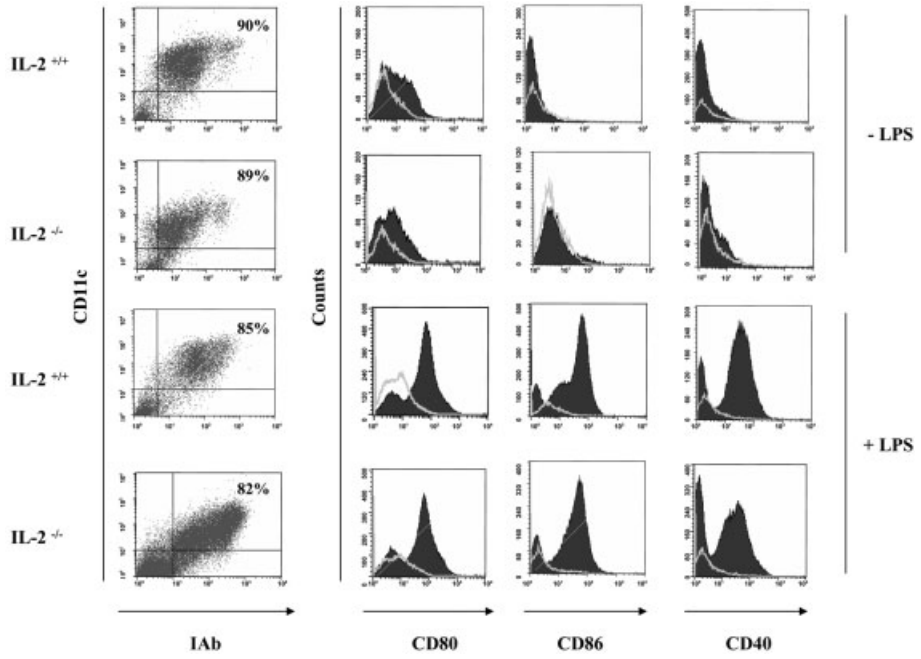

Figure 3. BM-DC differentiation from IL-$2^{-/-}$ BM progenitors. The phenotype (I-Ab, CD11c, CD40, CD80, CD86) of the *in vitro* generated BM-DC derived from WT and IL-$2^{-/-}$ mice in the immature state, and after 24 h of LPS stimulation (10 μg/mL) was assessed by flow cytometry analysis as described in Material and methods. Staining using isotype control mAb are depicted with empty grey curves and with relevant mAb (anti-CD80, -CD86, -CD40) in solid black curves.

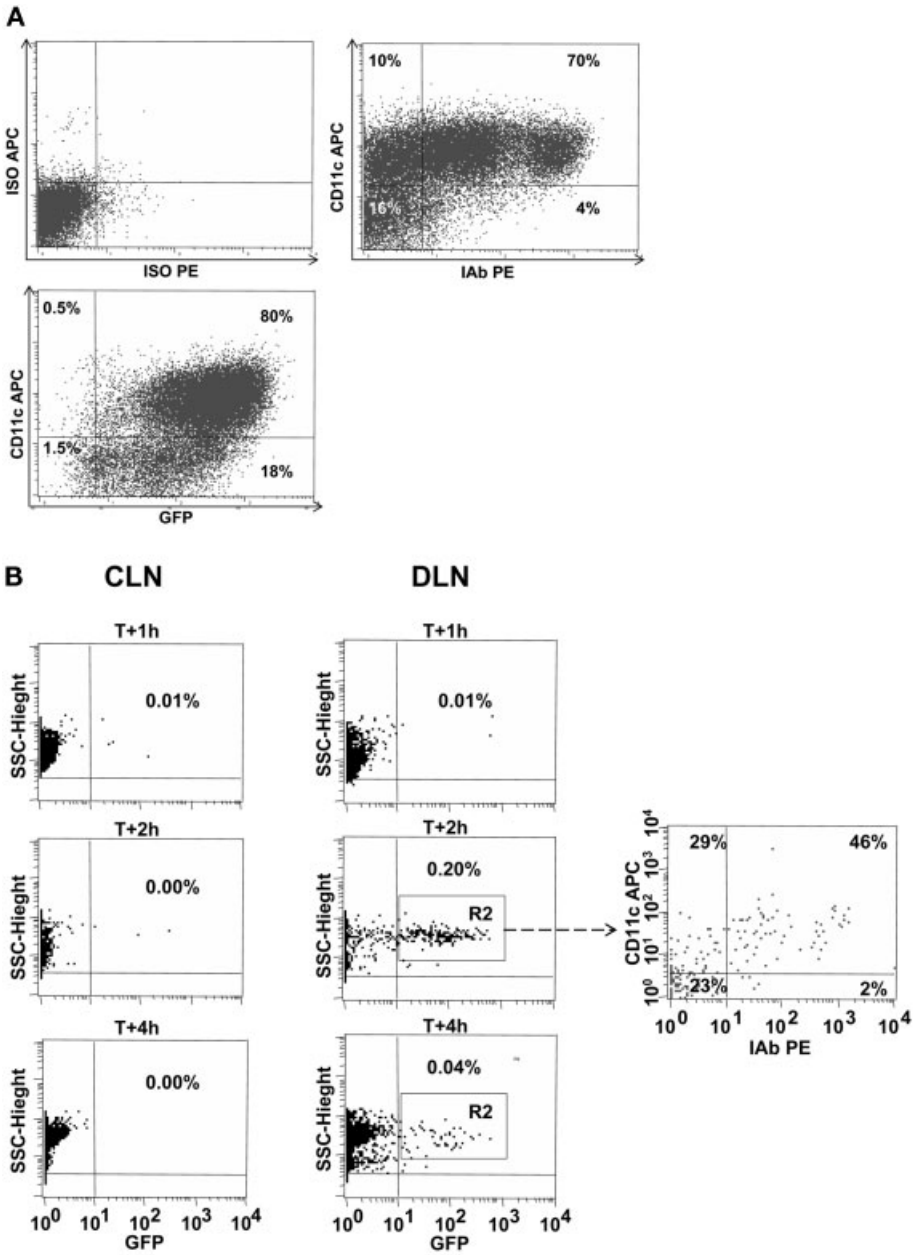

Figure 4. BM-DC stimulated for 2 h in LPS can reach the DLN within 2 h. BM-DC were propagated from eGFP-transgenic FM131 mice as described in Material and methods. (A) The culture of BM-DC^{GFP} was analyzed at day 13 in flow cytometry using anti-CD11c-APC and anti-I-A^b-PE mAb. (B) BM-DC^{GFP} were activated for 2 h in LPS and washed three times prior to footpad inoculation. Each C57BL/6 mouse received 3×10^5 BM-DC^{GFP} cells in one footpad and 50 μl of PBS in the contralateral one. Both, popliteal DLN and CLN cells, were harvested at 1, 2 and 4 h after footpad inoculation, enumerated in trypan blue exclusion and analyzed in flow cytometry after staining with anti-CD11c-APC, anti-I-A^b-PE mAb. Three mice per time points were analyzed and the results of a significant mouse are depicted.

promote a significant expansion of CD3$^-$/NK1.1$^+$ NK cells in the DLN compared with contralateral LN (CLN) (12 859 ± 9754 cells in DLN versus 1202 ± 719 cells in CLN, $p<0.01$, Fig. 7) at 24 h. Moreover, ^{2hLPS}DC IL-2$^{-/-}$ elicited activation of 32 ± 10% of NK cells residing in the DLN, while CLN contained less than 15% of CD69$^+$ NK cells (Fig. 7C, $p<0.01$). Immature DC did not allow any proliferation or any activation of NK cells (not shown). Therefore, both IL-2$^{-/-}$ and IL-2$^{+/+}$ BM-DC could induce LN-resident NK cell activation within 24 h after a 2-h stimulation with LPS.

Discussion

In response to danger signals, DC undergo a complete reprogramming, leading to markedly enhanced T cell and NK cell stimulatory capacities. The recent observations, that DC produce IL-2, early after microbial stimulation, opened up new molecular mechanisms in the regulation of immune responses by DC. Only microorganisms or microbial products seem to be able to induce the transcription of the IL-2 gene in DC, but not in macrophages [6, 11]. The adjuvant role of IL-2 produced by DC has been suggested because of the impaired ability of early activated, IL-2-deficient DC to induce primary alloreactive CD4$^+$ and CD8$^+$ T cells *in*

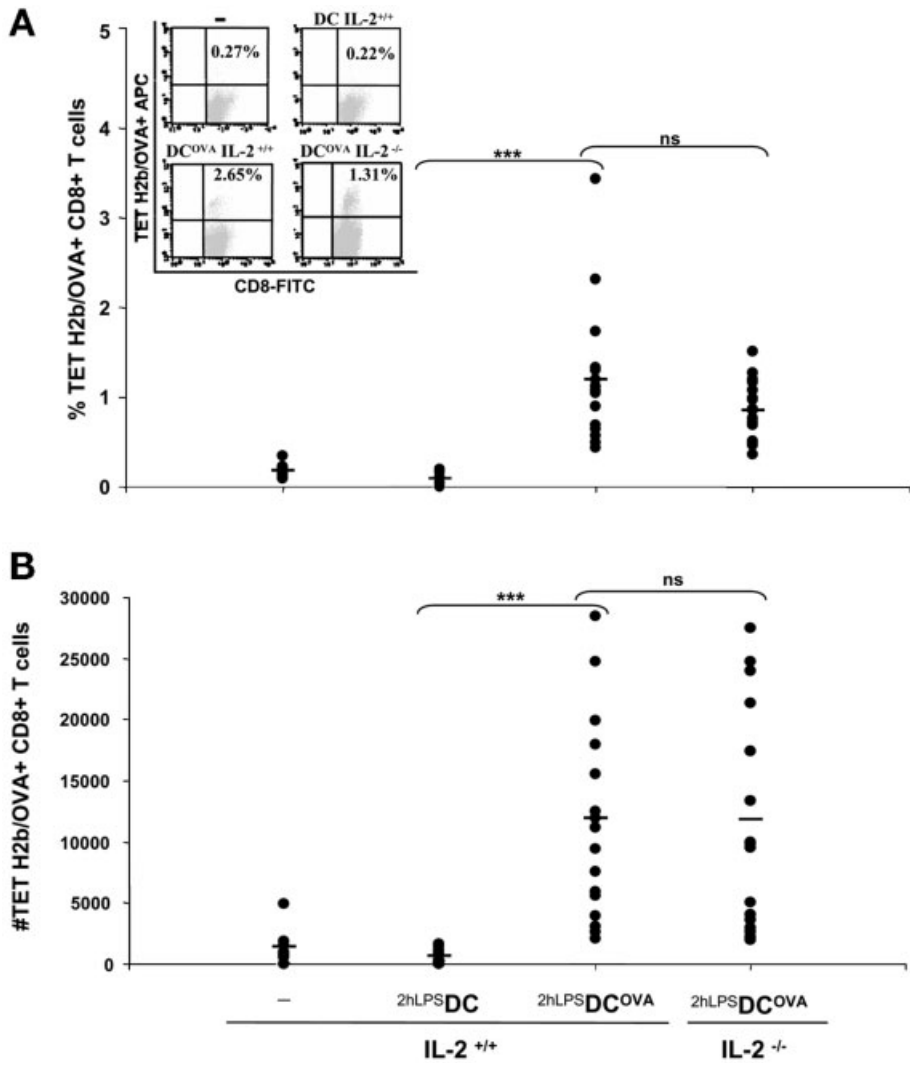

Figure 5. Expansion of OVA peptide-specific CTL following adoptive transfer of IL-$2^{+/+}$ and IL-$2^{-/-}$ BM-DC pulsed with OVA. At day 0, C57BL/6 mice were immunized in the footpad with 3×10^5 IL-$2^{+/+}$ (WT) and IL-$2^{-/-}$ BM-DC stimulated for 2 h with LPS and pulsed ($^{2hLPS}DC^{OVA}$) or not ($^{2hLPS}DC$) with OVA peptide. At day 5, mononuclear cells of the DLN were examined for the staining with H-2^b/OVA or H-2^b/TRP2 soluble fluorescent tetramers in flow cytometry. The percentages (A) and the absolute numbers (B) of tetramer-positive cells in the gated $CD3^+/CD8^+$ cells are shown. Graphs depict data obtained in three independent experiments. Each dot represents one mouse (n=18). *Statistically significant differences at 0.05 confidence interval (** at 0.01 and *** at 0.001) using Fisher's exact method; ns: not significant at 5% confidence interval. Dot plot analyses in flow cytometry after staining with H-2^b/OVA soluble fluorescent tetramers are shown in the insets in gated $CD3^+/CD8^+$ T cells, at day 5, in LN stimulated by PBS (–), DC, DC^{OVA} activated for 2 h with LPS of IL-$2^{+/+}$ or IL-$2^{-/-}$ mice.

vitro [11]. In addition, MCMV-infected DC induce a functional paralysis caused by their inability to prime immune responses [2]. At the early stage of the infection, the infected DC lose their capacity to secrete IL-2. However, our experiments conducted in naive C57BL/6 mice using the immunodominant OVA epitope (SIINFEKL) pulsed on IL-2 loss of function DC ruled out a critical role for early IL-2 production in the priming of $CD8^+$ Tc1 lymphocytes. The hypothesis of a passive transfer of antigenic material from adoptively administered DC to host APC (Fig. 6B) has been excluded by our experiments.

The role of IL-2 for T cell survival, antigen-driven proliferation, and generation of memory has been evaluated for $CD4^+$ and $CD8^+$ T cells. Using monoclonal populations of antigen-specific $CD4^+$ T cells, O. Lantz *et al.* [12] found that naive T cells cannot survive without the γ chain (γc) (used for IL-2, IL-4, IL-7, IL-9 and IL-15 receptor signaling), whereas memory T cells show no such requirement. However, neither naive nor memory γc-deficient T cells were impaired in their ability to proliferate and secrete cytokines in response to *in vivo* antigenic stimulation. A number of studies have documented that exogenous cytokines influence the

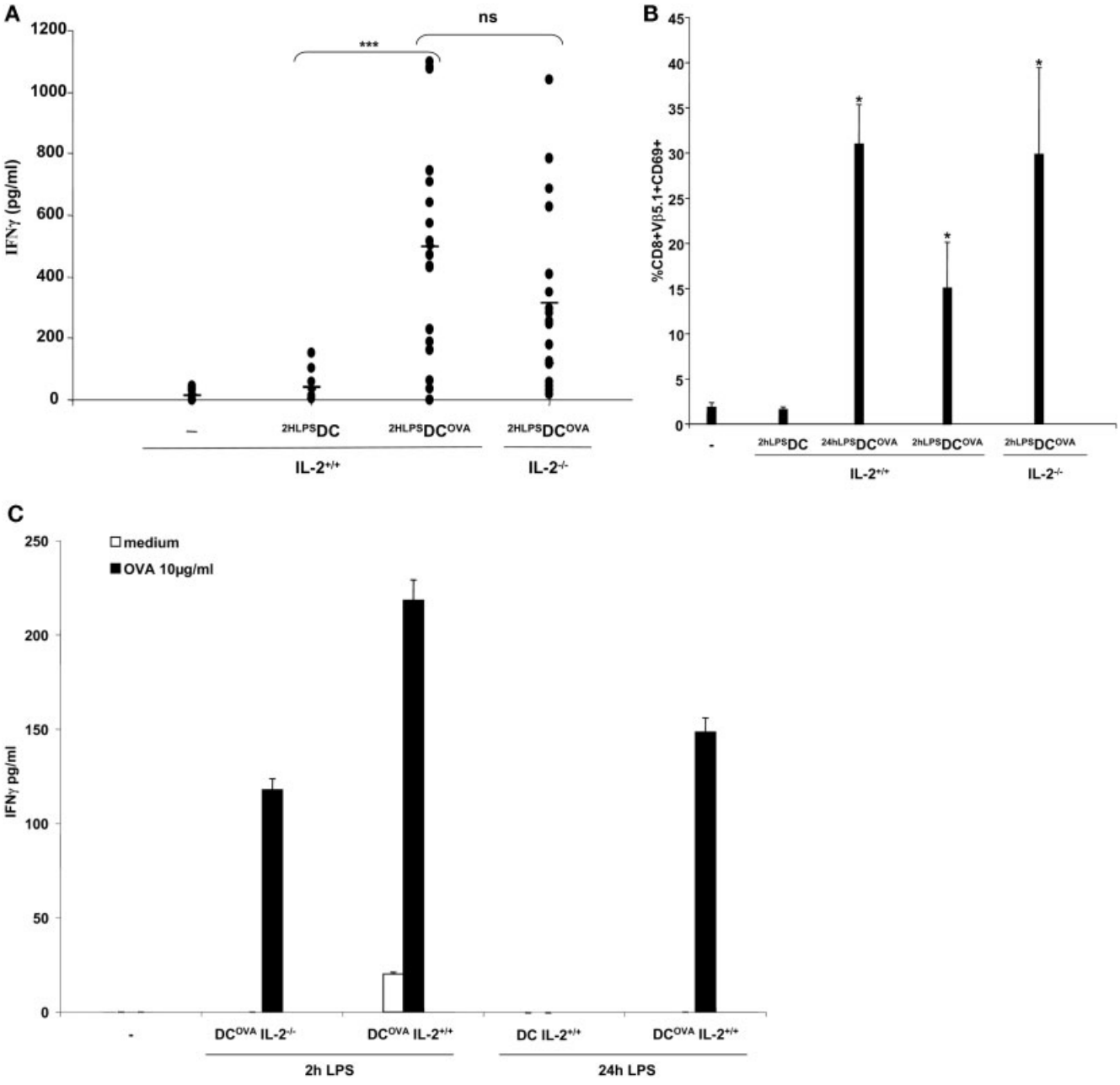

Figure 6. The priming and memory responses of Tc1 are not impaired using IL-$2^{-/-}$ BM-DC. (A) Same experimental setting than in Fig. 4. On day 5, mononuclear cells of the DLN corresponding to various immunizations were examined for IFN-γ secretion in 48–72-h *in vitro* cultures (containing 1% mouse serum) following stimulation with 10 μM OVA peptides. IFN-γ secretion was assessed using ELISA kits. Graphs represent data obtained in three independent experiments. Each dot represents one mouse (n=18). *Statistically significant differences at 0.05 confidence interval (** at 0.01 and *** at 0.001) using Fisher's exact method; ns: not significant at 5% confidence interval. (B) On day 0, an adoptive transfer of 10^6 OT-1 $CD8^+/V\beta5.1^+$ cells into β_2-microglobulin$^{-/-}$ mice was performed i.v. On day 1, 3×10^5 BM-DC propagated from IL-$2^{+/+}$ (WT) or IL-$2^{-/-}$ transgenic mice, pulsed (DC^{OVA}) or not (DC) with OVA peptides and activated by LPS for 2 h (^{2HLPS}DC) or for 24 h (^{24HLPS}DC, not producing IL-2 anymore in WT cells) were inoculated into the footpad. The monitoring of specific T cell activation was performed using flow cytometry 18 h later (staining with anti-CD3-CyC, anti-CD8-APC, anti-Vβ5.1-PE, anti-CD69-FITC). (C) C57BL/6 mice were immunized s.c. on day 0, with either 3×10^5 BM-DC pulsed or not with OVA peptides, and exposed to LPS for 2 h or 24 h and derived from IL-$2^{+/+}$ or IL-$2^{-/-}$ animals. After 5 weeks, animals were rechallenged (in the footpad) with 3×10^5 mature BM-DC pulsed with OVA (treated by 10 μg/mL LPS for 24 h). The capacity of LN monuclear cells to produce IFN-γ in response to OVA peptide *in vitro* was monitored 36 h later. A representative experiment using five mice per group out of two experiments is shown for (B) and (C). Means ± SEM are depicted on the graphs.

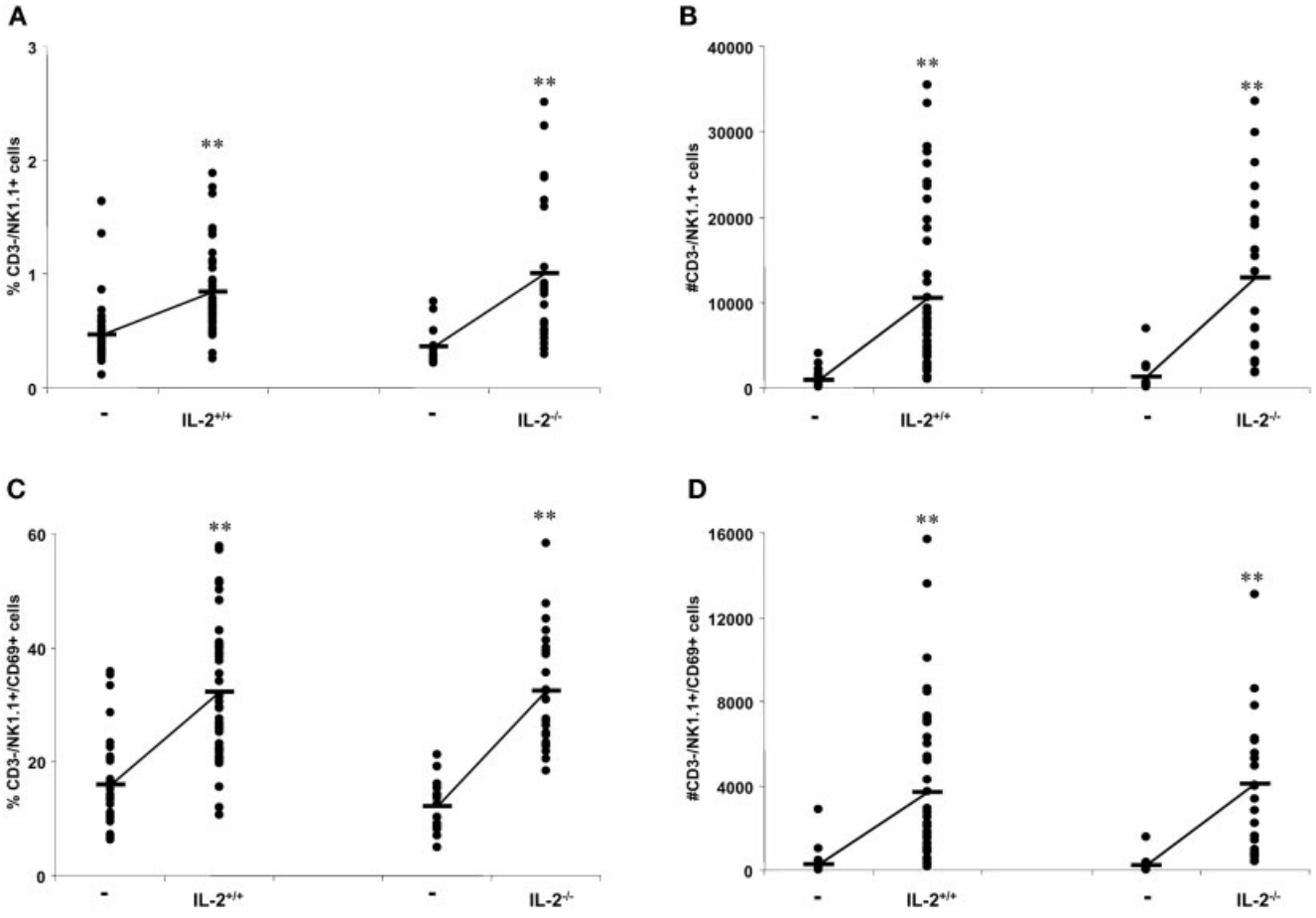

Figure 7. NK cell triggering in LN is not hampered using IL-$2^{-/-}$ BM-DC. C57BL/6 mice were immunized on day 0 in the footpad with 3×10^5 BM-DC of IL-$2^{+/+}$ and IL-$2^{-/-}$ mice. At 18 h, mononuclear cells of the popliteal DLN were examined for recruitment and activation of NK cells by FACS analysis. The percentages and the absolute numbers of NK cells ($CD3^-/NK1.1^+$) (A, B) and of activated NK cells ($CD3^-/NK1.1^+/CD69^+$) (C, D) cells are shown. Graphs depict data obtained in six independent experiments. Each dot represents one mouse (n=22). *Statistically significant differences at 0.05 confidence interval (** at 0.01 and *** at 0.001) using Fisher's exact method; ns: not significant at 5% confidence interval.

magnitude and quality of the resulting $CD8^+$ T effector CTL generated *in vitro* [13, 14]. In the absence of IL-2R and IL-4R signaling, TCR-activated $CD8^+$ T cells failed to differentiate *in vitro* into CTL, in part due to the lack of expression of granzyme B. They exhibited limited proliferation before apoptosis for the vast majority of the cells [15]. A critical role for IL-2 has also been shown during the *in vitro* generation of effector CTL for the optimal *in vivo* persistence of memory T cells. Signaling through the IL-2R is required for the development of $CD8^+$ T memory cells from naive normal antigen-specific precursor T cells [16]. Another study previously showed that proliferation requirements of effector-type $CD8^+CD45RA^+CD27^-$ T cells are different from those of the memory type in that the former population only divide in the presence of helper cytokines, such as IL-2 and IL-15 [17]. Furthermore, IL-4 has been shown by us and others [18] to prevent DC from responding to LPS for IL-2 secretion. Several reports have shown that the absence of IL-4, at the time of activation of CTL, increases the frequency of $CD8^+$ memory cells and enhances the effector functions of these memory cells [19, 20], supporting a potential role for IL-2 produced by DC precursors in CTL expansion and differentiation into memory T cells. However, our own study do not support a role for IL-2 produced by DC at the early phase of T cell priming for the generation of memory CTL responses (Fig. 6C).

We also learned from a survey performed by many groups on IL-2-deficient mice that autoimmune disease development (hemolytic anemia, inflammatory bowel disease) is rare under germ-free or gnotobiotic conditions or following introduction of a transgenic T cell receptor, suggesting that disease is not due to an inability of the IL-2-deficient host to fight opportunistic infections [21, 22]. Therefore, it is likely that IL-2 might be needed at the effector phase of $CD8^+$ T cell differentiation, while such cells are residing in the

periphery at the sites of ongoing inflammation (rather than at the priming phase in the LN).

DC have been shown to be instrumental for NK cell activation relevant against tumor development [9] and viral replication [23]. However, the molecular mechanisms underlying the DC-mediated NK cell lytic activity and IFN-γ production have not yet been fully elucidated. In mice, Terme *et al.* [8] showed a role for TREM2/KARAP DAP12 signaling in BM-DC for efficient NK cell triggering using BM-DC propagated exclusively in the presence of IL-4. Most of the reports alluded to the contribution of a cell to cell contact and/or IL-12 for IFN-γ secretion. In our recent study [24], we showed that DC/NK cell synapse formation is required for NK cell signaling and that IL-12 produced by LPS-activated DC is indispensable for NK cell IFN-γ production. However, IL-12 is not sufficient to promote cytolytic functions displayed by NK cells co-cultured with BM-DC. [24]. In mouse settings, LPS activation of mouse BM-DC propagated in the absence of IL-4 promotes IL-2 production, but only IL-15 production when IL-4 is introduced in the DC cultures [25]. The recent report of Granucci *et al.* [26] supports the notion that IL-2 produced by myeloid mouse DC after stimulation by *E. coli* is required for IFN-γ secretion by NK cells and the role of IFN type I in inducing NK cell cytolytic activity. The same authors show a relevance of IL-2 produced by DC in NK cell IFN-γ production (specifically in mice treated with anti-Thy-1 neutralizing mAb or in $Rag^{-/-}$ mice), necessary for the control of bacterial proliferation and metastatic dissemination *in vivo*. In the study by Granucci *et al.* [26], NK cell activation was assessed at very early time points (2–4 h following i.v. inoculation of *E. coli*), in flow cytometry using intracellular staining with anti-IFN-γ mAb in splenic NK cells, most experiments being performed in $Rag^{-/-}$ mice.

The main difference between our study and that of Granucci *et al.* [26] concerning NK cells resides mainly in the time point of investigation, and, if IL-2 seems to be required very early at the onset of infection for NK cell activation, it is not indispensable for late NK cell recruitment and proliferation (Fig. 7). Secondly, we have investigated NK cell numbers and up-regulation of CD69 molecules, while Granucci *et al.* have monitored NK cell IFN-γ secretion. It is noteworthy that some authors [27] underscored that the mechanisms involved in up-regulation of CD69 molecules are not identical to that required for NK cell IFN-γ or cytotoxicity [role of TNF-α for the former, role of IL-12 (or IL-2 in the absence of IL-4) and IFN type 1 for the latter, respectively]. Thirdly, we have reported (Ghiringhelli F *et al.*, paper submitted) that naturally occurring $CD4^+CD25^+$ regulatory T cells (Treg) inhibit NK cell effector functions *in vitro* and *in vivo*, directly via TGF-β and indirectly by interfering with DC functions. In $Rag^{-/-}$ mice [26], naturally occurring Treg are lacking and, therefore, IL-2 produced by DC might be more beneficial to NK cells, suggesting that the high-affinity IL-2R harbored by Treg might override that of NK cells. We are currently investigating whether IL-2 produced by DC might trigger Treg proliferation *in vivo*.

However, other studies suggested a role for IL-2 produced by $CD4^+$ T cells in the activation of bystander LN NK cells [28]. In the CT26 tumor model where DC primed NK cells for tumor rejection, $CD4^+$ T cells were required for induction of the NK cell anti-tumor response [29]. From our data addressing the role of IL-2 produced by DC themselves for LN NK cell activation (using the early activation marker CD69), we could not determine whether this was involved in the DC-mediated NK cell activation in LN.

Finally, the report by Kronin *et al.* [30] did not indicate a role for the inducible IL-2Rα on DC for DC development, for T cell stimulation or for regulation of T cell responses. Our data do not support the critical part of early IL-2 production by mouse BM-DC stimulated in LPS in the DLN for either T cell priming or NK cell activation, but does not rule out its potential relevance at ports of entry for T cell reactivation. The demonstration of IL-2 production by human DC will possibly shed some light on the pathophysiology of this intriguing biological feature [31].

Material and methods

Mice

Female WT C57BL/6 (H-2^b) and WT BALB/c (H-2^d) were obtained from the Centre d'Elevage Janvier (Le Genest St Isle, France), the Centre d'Elevage Iffa Credo (L'Albresle, France) and maintained in our animal facility according to the Animal Experimental Ethics Committee Guidelines. IL-2-deficient mice and WT littermates [32] were kindly provided by A. Schimpl (University of Würzburg, Germany), the green mice FM131 (C56BL/6 TgN(act-EGFP)osbC14-Y01-FM131) by M. Okabe (University of Osaka, Japan) [33] and the OT-1 mice by B. Combadière (U543 INSERM, Paris, France). The β_2-microglobulin$^{-/-}$ mice were bred in the animal facility of the Institut Pasteur, Paris and used after the sixth backcross generation onto C57BL/6 mice. All experiments were performed in compliance according to relevant laws and institutional guidelines. All mice were used at 6–25 weeks of age.

BM-DC culture conditions for IL-2 production

Mouse BM-DC were cultured as previously described [34]. Briefly, BM progenitor cells were grown in IMDM culture medium (Sigma, France) supplemented with 50 IU/mL penicillin, 50 μg/mL streptomycin, 2 mM L-glutamine, 10% decomplemented fetal calf serum (Gibco-BRL, France), 50 μM

2-ME (Sigma) and 30% J558-mGM-CSF 13-day culture supernatants. Alternatively, BM-DC were propagated from BM progenitor cells in culture medium supplemented with 1000 IU/mL rmGM-CSF (R&D Systems, INC. Minneapolis, MN) and with 1000 IU/mL rmIL-4 or 10 ng/mL rmIL-13 (R&D Systems) as previously described [35]. Culture medium was renewed every 2 days and BM-DC were used on day 6. DC cultures considered for the experiments contained 90 ± 8% of $CD11c^+$ DC. The phenotype of BM-DC was analyzed by flow cytometry using anti-mouse CD11c, $I\text{-}A^b$, CD80, CD86 and CD40 mAb (Pharmingen, Heidelberg, Germany). Activation of BM-DC was induced by adding increasing dosages of LPS (0.001–100 μg/mL; Sigma) to culture medium for 24 h. Alternatively, activation of BM-DC was induced with 10 μg/mL endotoxin-free CpG oligomeric sequences (5′-TCCAT-GACGTTCCTGACGTT-3′), with poly [I]:poly $[C_{12}U]$double-stranded RNA (Ampligen® 2.5 mg/mL, Bioclone (Pty) Ltd., Republic of South Africa, provided by M. Adams, Velindre Hospital, Cardiff, UK), with flagellin (100 μg/mL), or with TNF-α (20 ng/mL, Abcys, France). Supernatants of activated BM-DC were harvested at serial time points up to 24 h, stored at –80°C. Supernatants of BM-DC cultures stimulated for increasing amounts of hours (kinetic studies of IL-2 production by BM-DC) were collected and stored at –80°C before dosage.

IL-2 ELISA

ELISA was performed using the BD OptEIA ELISA Set (Pharmingen) and following the manufacturer's recommendations. The sensitivity of the mIL-2 kit was >3.1 pg/mL. We depleted the BM from contaminating $CD3^+$ T cells and used growth factors (such as GM-CSF), which do not favor T cell differentiation and consequently IL-2 production by T cells.

CTLL-2 bioassay

CTLL-2 were seeded at 2×10^5 cells/96 well plates in U-bottom plates in the presence of increasing dosages of rhIL-2 or supernatants of BM-DC stimulated with LPS. Proliferation of CTLL-2 was measured by incorporation of $[^3H]$thymidine, after a pulse, at day 5.

CTL activation *in vivo*

Each experimental groups contained three to five mice, and experiments were performed at least three times. Pooled data of the experiments are shown on the graphs. BM-DC – at a concentration of 10^6/mL – were loaded with SIINFEKL synthetic peptides at 10 μM for 2 h during LPS stimulation at 37°C, 5% CO_2. Mice were immunized once, at day 0, with 3×10^5 day-13 $BM\text{-}DC^{OVA}$ in the footpad of mice. At day 5, popliteal DLN and CLN were harvested, and cells were enumerated using trypan blue exclusion assays. LN cells were first stained with 0.2 μg $H\text{-}2^b$/OVA or control fluorescent (APC) soluble tetramers (Beckman Coulter, Immunomics, France) for 30 min at room temperature in 20 μl PBS 1× 0.5% BSA (Sigma), then with anti-CD3-FITC and anti-CD8-PE mAb (Pharmingen) for 30 min at room temperature prior to washing steps and analysis in a FACSCalibur® (Becton Dickinson, Heidelberg, Germany). Function of LN-residing T cells following immunization was assessed by challenging LN mononuclear cells with grading doses of OVA peptides. Supernatants were collected after 48–72 h to evaluate IFN-γ levels (BD OptEIA ELISA, Pharmingen). For the study of memory T cell responses, C57BL/6 mice were immunized s.c. at day 0 with 3×10^5 BM-DC. Ater 5 weeks, animals were rechallenged (in the footpad) with 3×10^5 mature WT BM-DC pulsed with OVA (treated by 10 μg/mL LPS for 24 h). The capacity of LN monuclear cells to produce IFN-γ in response to OVA peptide *in vitro* was monitored 36 h later, using ELISA kits.

LN analyses of NK cells

Processes are detailed in figure legends. Briefly, following three washing steps in PBS, 3×10^5 day-13 BM-DC stimulated with 10 μg/mL LPS for 2 h, were inoculated in one footpad of C57BL/6 mice. Popliteal ipsi- and contralateral LN were harvested at 28 h. LN mononuclear cells were mechanically minced with or without collagenase and DNAse usage. Cells were enumerated using trypan blue exclusion prior to three-color immunostaining with mAb for FACS analysis (anti-CD3-FITC, anti-NK1.1-APC, anti-CD69-PE).

Statistical analyses

Tetramer $H\text{-}2^b/OVA^+$ $CD8^+$ T cells, IFN-γ levels and NK cells have been analyzed using an ANOVA analysis of variance with Fisher's exact method. Significances within 95%, 99% and 99.9% confidence interval are depicted on the graphs with *, ** and ***, respectively. Comparisons between groups were performed using the non-parametric Kruskal-Wallis test.

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