 DOI 10.1002/eji.200838659 Eur. J. Immunol. 2008. 38: 2776–2783

European Journal of Immunology

SHORT COMMUNICATION

# A novel CD11c.DTR transgenic mouse for depletion of dendritic cells reveals their requirement for homeostatic proliferation of natural killer cells

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**Dendritic cells (DC) are known to support the activation of natural killer (NK) cells. However, little is known about the role for DC in NK-cell homeostasis. In order to investigate this question, a novel bacterial artificial chromosome transgenic mouse model was generated in which the diphtheria toxin receptor is expressed under the CD11c promoter. In these mice efficient DC depletion can be achieved over prolonged periods of time by multiple injections of diphtheria toxin. We show here that NK cells require DC for full acquisition of effector function *in vivo* in response to the bacterial-derived TLR ligand CpG. Importantly, DC were found to play an instrumental role for maintaining normal homeostasis of NK cells. This is achieved by IL-15 production by DC, which supports the homeostatic proliferation of NK cells.**

**Key words:** Dendritic cells · Diphtheria toxin receptor · Homeostasis · Natural killer cells

Supporting Information available online

## Introduction

Natural killer (NK) cells play an important role in the innate host defence against pathogens and tumours [1]. There is much known about the molecular mechanisms of NK-cell functions, but the factors influencing NK-cell numbers are only beginning to be elucidated. Mature NK cells were typically thought to be a terminally differentiated population, with a very limited self-renewal capacity. However, it was recently shown that a small percentage of NK cells actively proliferate in the steady state, resulting in a half life of the NK-cell population of about 17 days [2]. In addition, like B and T lymphocytes, it was recently found that NK cells can undergo homeostatic proliferation in mice with a reduced NK-cell compartment [2, 3]. While IL-7 is essential for the generation and expansion of the T-cell compartment [4–6], IL-15 appears to play a dominant role in NK-cell proliferation and survival [2, 7]. Mice deficient in IL-15 or IL-15R lack peripheral NK cells [8, 9], while mice transgenic for IL-15 show a dramatic increase in NK-cell numbers [10, 11].

NK-cell cross talk with dendritic cells (DC) has been described *in vitro*, resulting in activation of both NK cells and DC [12]. It is known that DC contribute to T-cell homeostasis, especially under lymphopenic conditions [13], but a role for DC in NK-cell homeostasis has not been investigated. In order to study the interactions between DC and NK cells *in vivo* we utilize here a new bacterial artificial chromosome (BAC) transgenic CD11c.DTR mouse model, designated CD11c.DOG, which allows effective depletion of DC over prolonged periods of time without non-specific cytotoxicity. Using this model, we show that DC are required for NK-cell activation *in vivo* in response to TLR ligands.

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Importantly, we observe a previously unrecognized role for DC in optimal homeostatic proliferation of NK cells in lymphopenic conditions. In this process, DC-derived IL-15 appears to play an important role. Our data indicate that not only T lymphocytes, but also NK cells require DC for homeostatic proliferation.

## Results

### CD11c.DOG mice allow long-term ablation of DC *in vivo* without toxicity effects

In order to study the effect of *in vivo* interaction between DC and NK cells, a system that permits long-term depletion of DC is required. The diphtheria toxin/diphtheria toxin receptor (DT/DTR) system introduced by Saito *et al.* [14] has been successfully used for depletion of various cell types *in vivo* [15–18]. In previously published CD11c.DTR mice, the DTR is under the control of a cloned CD11c promoter [18]. Although these mice allow successful depletion of DC following a single injection of DT, multiple injections of DT lead to death unless bone-marrow chimeras are made, possibly due to aberrant expression of the DTR transgene [18, 19]. In order to achieve faithful gene expression, we developed a novel CD11c.DTR transgenic mouse line that would allow prolonged DC depletion. For this purpose, we cloned the DTR (together with a fragment of the ovalbumin protein and eGFP) into a BAC under the control of the full CD11c promoter (Fig. 1A). In this particular mouse strain, designated CD11c.DOG, the eGFP component of the fusion protein failed to give a fluorescence signal, whereas the ovalbumin protein was expressed in DC, resulting in extensive proliferation of ovalbumin-specific CD4 (OT-II) and CD8 (OT-I) cells after transfer into the CD11c.DOG mice (data not shown).

Injection of 8 ng/gram body weight (gbw) DT induced depletion of DC ($CD11c^{high}$ MHC class $II^+$) in spleen, lymph nodes, thymus and bone marrow, albeit efficiency of depletion varied in the different organs tested (Fig. 1B and C). The efficiency of depletion was consistently highest in the spleen, followed by thymus, lymph nodes and bone marrow, even after administration of high amounts of DT (64 ng/gbw) (Fig. 1C). This was also observed after intravenous injections of DT (data not shown). Differential access of DT to the spleen compared with other organs after intraperitoneal or intravenous injections may account for the differences in depletion efficiency observed. DT had no effect on the number or percentage of DC in wild type B6 mice (data not shown). Depleted mice regenerated DC and filled up their DC compartment to the original number in about 3–4 days (Fig. 1D). Thus, for long-term studies continuous depletion of DC by multiple injections of DT is required.

The data shown in Fig. 1E confirm that the previously published CD11c.DTR mice do not survive multiple injections of 4 ng/gbw DT [18]. In contrast, we found that daily 8 ng/gbw DT administrations to CD11c.DOG mice did not result in reduced survival or weight loss (Fig. 1E). However, there is an upper limit of the DT dose that can be applied, as daily injections of high amounts of DT (64 ng/gbw) were toxic to both B6 and CD11c.DOG mice, leading to weight loss and death of mice (Supporting Information Fig. 1). The exact dose resulting in weight loss of B6 mice varied with different batches of DT, but amounts greater than 35 ng/gbw consistently resulted in weight loss of mice (data not shown). Since 8 ng/gbw effectively depleted $>90\%$ of DC in the spleen, we decided to use this amount for most further experiments.

Although mice tolerated well 8 ng/gbw for prolonged periods of time, DC depletion was limited to a period of 11–12 days (Fig. 1F). In order to see, if the loss of effective DC depletion from day 13 onwards was due to formation of neutralizing antibodies against DT, we analysed sera from B6 and CD11c.DOG mice treated every other day for 3 wk with DT. Using a DT-specific ELISA we observed that these sera contained antibodies against DT (data not shown). Importantly, these sera prevented the RMA.DTR cell line from being killed *in vitro* by DT (Fig. 1G). Thus, prolonged injections of DT resulted in the formation of neutralizing antibodies, although most DC had been depleted in these mice. In order to circumvent the problem of neutralizing antibodies, we have generated a transgenic mouse that is tolerant to DT (K. Hochweller *et al.*, unpublished).

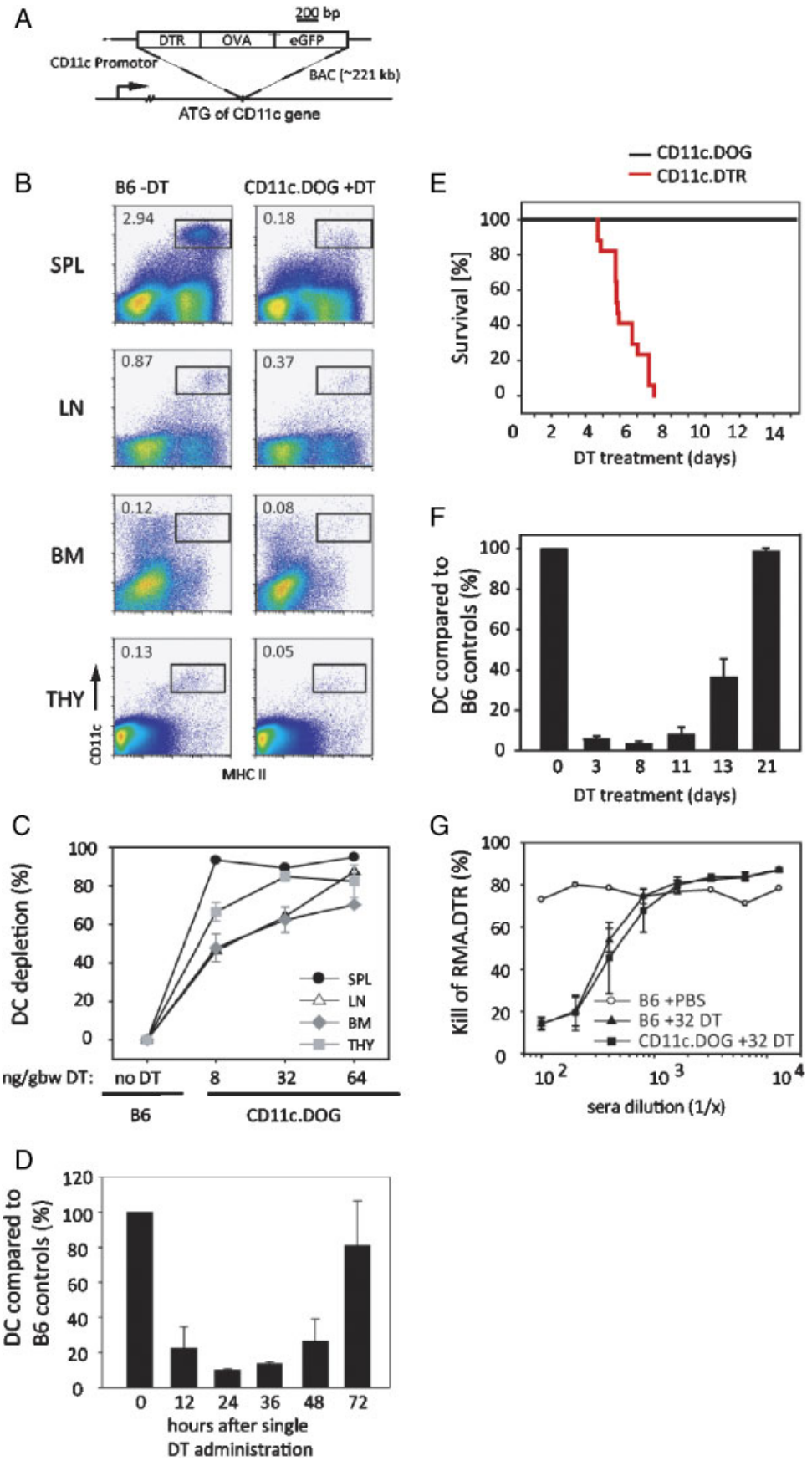
In order to confirm faithful expression of the BAC, we thoroughly examined CD11c expression and depletion of B cells, T cells, NK cells and NKT cells in spleens after 8 ng/gbw DT administration. The minor $CD11c^{hi}$ subpopulations of these cell types were effectively depleted, whereas $CD11c^{int/lo}$ cells were only partially depleted (Supporting Information Fig. 2A), probably due to lower expression of the DTR in these cells. Importantly, a considerable fraction of NK cells (about 16%) express intermediate levels of CD11c, but these cells were depleted minimally following 8 ng/gbw DT (Supporting Information Fig. 2A). Histology of spleen sections revealed that $F4/80^+$, $MOMA\text{-}1^+$ and $ERTR\text{-}9^+$ subpopulations of macrophages were also depleted in DT treated CD11c.DOG mice (Supporting Information Fig. 2B), similar to results obtained in the published conventional transgenic CD11c.DTR mice [19]. A so far unreported consequence of DT-mediated depletion of DC was the appearance of $Gr\text{-}1^+$ $CD11b^+$ cells in the spleen of treated CD11c.DOG (but not wt B6) mice, peaking in cell number at 2 days after DT administration, but rapidly returning to normal levels by the third day after DT treatment (Supporting Information Fig. 2B–D). We also observed this in the conventional transgenic CD11c.DTR mice (data not shown).

In conclusion, the new BAC transgenic CD11c.DOG mouse model introduced here allows effective long-term depletion of $CD11c^+$ cells.

### DC are required for optimal NK-cell activation and homeostatic proliferation *in vivo*

Next, we investigated, whether or not DC would support activation of NK cells by TLR ligands and homeostatic expansion



of NK cells. NK cells were activated by administration of the TLR-9 ligand CpG and the *in vivo* lytic activity determined by injection of CFSE-labelled tapasin-deficient splenocytes that served as NK targets. CpG administration in mice with a normal DC compartment lead to increased NK cytotoxicity *in vivo* (Fig. 2A, left panel). This enhanced activity was fully dependent on the presence of DC during CpG-mediated activation, thereby confirming a requirement for DC for NK-cell priming [20]. The absence of NK kill activity observed after DC ablation was not due to depletion of $CD11c^+$ NK cells, because the frequency and total number of NK cells was not altered after administration of CpG and DT (Fig. 2A, right panel, and Supporting Information Fig. 2A).

The homeostasis of T cells has been well studied and found to require several factors including the presence of DC, self MHC molecules and the cytokine IL-7 for proliferation and survival [21]. Much less is known about NK-cell homeostasis. It has been reported that MHC molecules do not play a role, but a requirement for DC has not yet been investigated. To investigate whether DC would be required not only for NK activation (Fig. 2A) but also for NK-cell homeostasis, mature $CD45.1^+$ NK cells were CFSE-labelled and transferred into normal or DC-depleted $CD45.2^+$ mice rendered lymphopenic by sublethal irradiation. Since DC return to normal levels within about 3–4 days after depletion, long-term depletion of DC was required for this experiment. Seven days after transfer and daily DT application, recipient mice were sacrificed, and CFSE profiles, percentages and numbers of donor-derived NK cells were determined by flow cytometry using CD45 congenic markers. After 7 days of homeostatic proliferation, we consistently observed fewer cell divisions of mature donor-derived NK cells ($NK1.1^+$ $CD3^-CD45.1^+$) in DC-depleted CD11c.DOG mice compared with DC-sufficient B6 mice (Fig. 2B and C). Consequently, the number of recovered donor-derived NK cells was higher in B6 than in CD11c.DOG mice (Fig. 2D). The numbers of donor-derived NK cells recovered in lymphopenic B6 hosts were comparable to previously published data [2]. These data implicate DC as an important component in the homeostasis of mature NK cells in lymphopenic conditions.

## DC-derived IL-15 contributes to lymphopenia-induced proliferation of NK cells

The cytokine IL-15 has been reported to be crucial for activation and survival of NK cells [2, 7, 20], but its role for homeostatic proliferation of NK cells is less clear. One study reported that NK cells failed to proliferate after transfer into IL-15-deficient lymphopenic hosts [7], whereas another study found NK homeostasis to be largely independent of IL-15 [2]. In view of our above observation that DC supported NK-cell homeostasis, we investigated whether or not IL-15 production by DC was required for this process. For this purpose we created the mixed bone-marrow chimeras, depicted in Fig. 3A. Wt B6 or $IL\text{-}15^{-/-}$ bone marrow ($CD45.2^+$) was mixed with CD11c.DOG bone marrow ($CD45.1^+$) at a 1:1 ratio and used to generate chimeric mice in which DT application would result in a DC compartment consisting only of IL-15-sufficient or IL-15-deficient DC, respectively.

Mature NK cells from $CD45.1^+/CD45.2^+$ heterozygote mice (and thus distinguishable from endogenous NK cells derived from $CD45.2^+$ homozygote wt B6 or $IL15^{-/-}$ and $CD45.1^+$ homozygote CD11c.DOG bone marrow) were CFSE-labelled before transfer into sublethally irradiated and DT-treated chimera recipients. Seven days after transfer and continuous DT treatment, CFSE profiles, percentages and numbers of donor-derived NK cells were determined in recipient mice. The CFSE profiles revealed a significant reduction in the percentage of dividing NK cells in $IL\text{-}15^{-/-}$+CD11c.DOG chimeras as compared with B6+CD11c.DOG chimera controls (Fig. 3B and C), which amounted to an about 50% decrease (Fig. 3C). This was paralleled by a reduced recovery of transferred NK cells (Fig. 3D).

IL-15 can be expressed by a variety of cell types, including leucocytes and epithelial cells [22, 23], but our data indicate that DC are a particularly important source for the IL-15 required for homeostatic proliferation of NK cells. These results suggest that NK cells have to be in close contact or vicinity to DC in order to receive an optimal amount of IL-15. This assumption is in agreement with the observation that in lymph nodes NK

◀ **Figure 1.** CD11c.DOG mice for long-term ablation of DC *in vivo*. (A) Construct used to generate CD11c.DOG mice. A fusion construct consisting of the human DTR, an ovalbumin 140–386 fragment, and eGFP was cloned into a BAC under the CD11c promoter by homologous recombination. (B) DC depletion *in vivo* by DT administration in CD11c.DOG mice. Flow cytometry of splenocytes (SPL), lymph node cells (LN), bone-marrow cells (BM) or thymocytes (THY) 24 h after a single DT injection (8 ng/gbw). Numbers indicate percentage of $CD11c^{hi}$ MHC class $II^+$ conventional DC of live cells in the different organs. Representative FACS plots are shown. The experiment was repeated four times, $n = 3$–4 mice per group. (C) Dose-dependent depletion of DC in different organs in CD11c.DOG mice. Mice were treated with different doses of DT and 24 h later, organs were analysed as in (B). Shown is one representative of two to four experiments with four mice per group for each dose. Percentage depletion was calculated based on numbers of DC per spleen in DC-depleted and non-depleted mice. (D) DC recovery after DT administration. Kinetics of DC depletion and recovery in the spleen after a single DT injection ($n = 4$ for each time-point). Shown is one representative of three experiments. (E) Survival of BAC transgenic CD11c.DOG mice compared with conventional CD11c.DTR transgenic mice following multiple injections of DT. Mice received the indicated doses of DT every second day for 2 wk. Shown is one representative out of three experiments, CD11c.DOG $n = 17$, CD11c.DTR $n = 10$. (F) Continuous depletion of DC in the spleen following daily DT application. Mice received 8 ng/gbw DT daily. Bar graphs show the percentages of $CD11c^{hi}$ MHC class $II^+$ conventional DC of total live splenocytes of CD11c.DOG mice compared with B6 mice, after 0, 2, 7, 12 and 20 days of DT administration. Shown is one representative out of three experiments, $n = 4$ mice for each time-point. (G) Sera from DT treated mice contain protective anti-DT antibodies. RMA.DTR cells were incubated with $10^{-9}$ M DT together with serial dilutions of sera from mice treated for 21 days with DT. The percentage of RMA.DTR-eGFP lysis was calculated as described in Supporting Information *Materials and methods*.

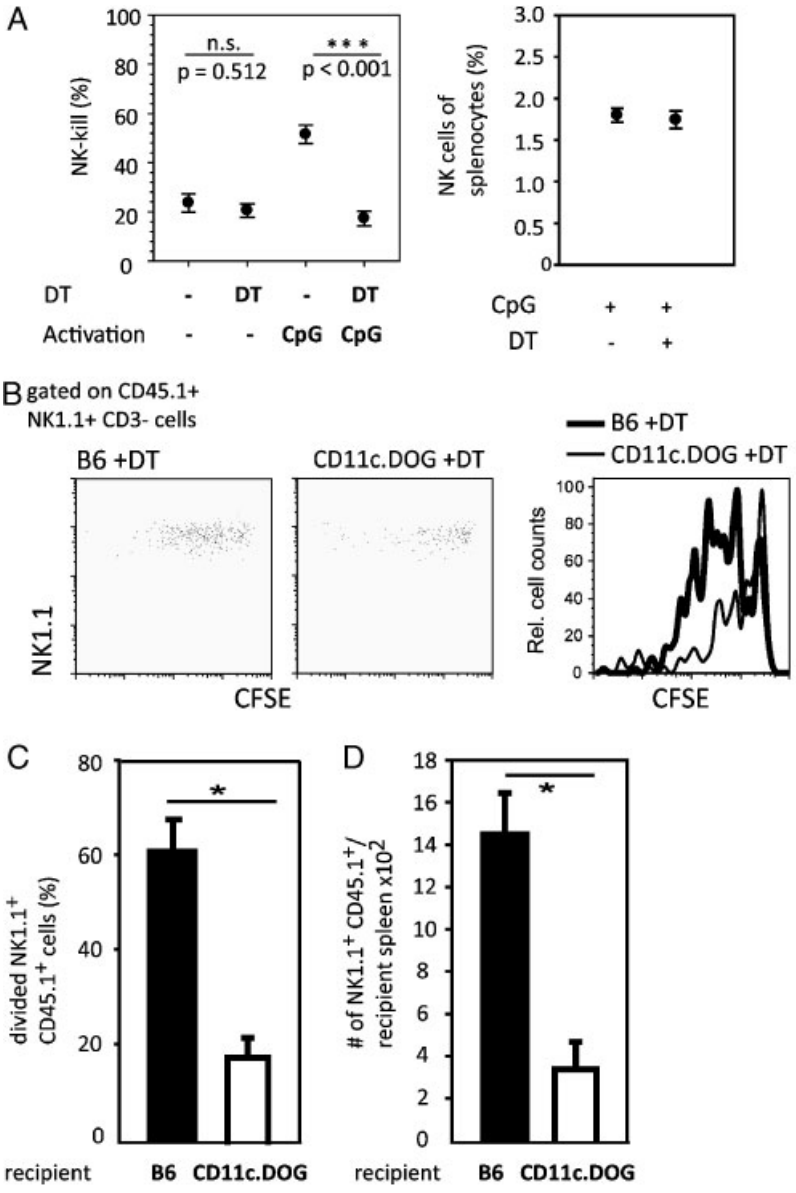



**Figure 2.** DC are required for optimal homeostatic proliferation of NK cells. (A) NK cells require DC for optimal NK kill activity *in vivo*. Left panel: Mice received 8 ng/gbw DT, followed 24 h later by 30 μg CpG. After another 24 h the percentage of NK-mediated kill *in vivo* was determined. Data are pooled from three independent experiments, $n = 8$–9 mice in total. Right panel: The percentage of NK cells in spleens was not changed following DT administration. Mice first received 30 μg CpG, followed by 8 ng/gbw DT 24 h later. Numbers of NK cells were quantified by flow cytometry 48 h later. The experiment was repeated twice with similar results, $n = 4$. (B–D) Decreased homeostatic proliferation and cell numbers of mature NK cells in absence of conventional DC. B6 or CD11c.DOG recipient mice were sublethally irradiated (6 Gy) 1 day prior to transfer of $1 \times 10^6$ CFSE-labelled DX-5$^+$ NK cells. Recipient mice received DT daily from 1 day before transfer of NK cells until the end of the experiment. (B) Representative CFSE profiles of NK1.1$^+$, CD45.1$^+$ NK cells 7 days after transfer into DT-treated B6 (left, and thick line on histogram on right) or CD11c.DOG mice (middle, and thin line on histogram on right). (C) The percentage of donor NK cells, which have undergone extensive cell divisions as indicated by loss of CFSE. (D) Numbers of transferred (CD45.1$^+$) NK cells were quantified 7 days after transfer into irradiated mice. Three mice were used in each group. The experiment was repeated three times with similar results. $^*p<0.05$, Student's *t*-test.

cells and DC form close contacts [24]. Stimulation of NK-cell proliferation *via* DC-derived IL-15 may also be relevant under physiologic conditions. For example, infections can increase IL-15 production by DC, which would locally (at the site of infection) stimulate NK-cell proliferation and acquisition of effector functions.

It needs to be emphasized that the homeostatic proliferation of NK cells observed here appears to depend only partially on DC and DC-derived IL-15, suggesting the existence of additional mechanisms that support NK homeostatic proliferation. This situation is reminiscent of T cells. However, consistent with differential dependency on MHC and cytokines, differences appear to exist between CD8 and CD4 T cells, CD8 T cells appearing to be more dependent on DC for homeostatic proliferation in lymphopenic hosts than CD4 T cells [13].

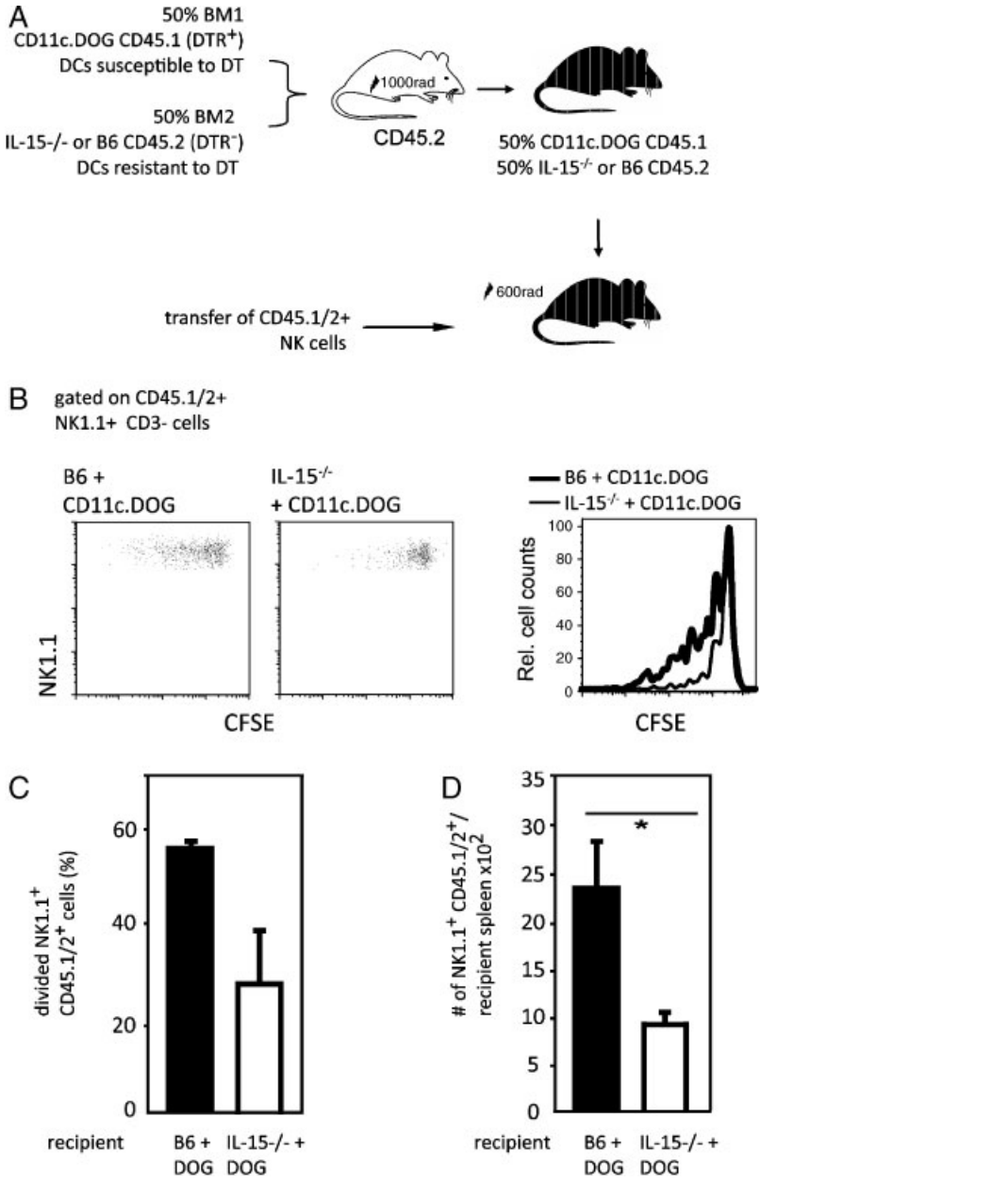



**Figure 3.** DC-derived IL-15 contributes to homeostatic expansion of mature NK cells. (A) Schematic representation of the mixed BM chimeras used in this study. See *Materials and methods* for a detailed description of the BM chimeras. (B–D) NK-cell proliferation and numbers are decreased after transfer into mice containing only IL-15$^{-/-}$ DC. Mixed bone-marrow chimeras shown in (A) were sublethally irradiated (6 Gy) 1 day prior to transfer of $1 \times 10^6$ CFSE-labelled DX-5$^+$ NK cells. Recipient mice received DT daily from 1 day before transfer of NK cells until the end of the experiment. (B) Shown are representative CFSE profiles of donor-derived NK1.1$^+$ CD3$^-$CD45.1/2$^+$ NK cells 7 days after transfer into DT-treated B6 (left, and thick line on histogram on right) or IL-15$^{-/-}$+CD11c.DOG mice (middle, and thick line on histogram on right). (C) Shown is the percentage of donor NK cells, which have undergone extensive cell divisions as indicated by loss of CFSE. (D) Numbers of transferred (CD45.1/2$^+$) NK cells were quantified 7 days after transfer into irradiated chimeras. Three mice were used in each group. The experiment was repeated three times with similar results. $^*p<0.05$, Student's *t*-test.

## Concluding remarks

In summary, this study introduces a new BAC transgenic CD11c.DTR mouse model for prolonged *in vivo* depletion of DC by application of DT, without any apparent signs of toxicity. Experiments using this mouse line demonstrated a previously unrecognized role for DC in the homeostasis of NK cells, and showed that local production of IL-15 by DC is required for the maintenance of the NK-cell compartment. Thus, the present study adds an additional function to the long list of DC functions, which so far encompass antigen presentation to and activation of CD4 and CD8 T cells, deletion of T cells and induction of regulatory

T cells, and support of T-cell homeostasis. The requirement of DC for NK-cell homeostasis described here emphasizes again the central role of DC for both adaptive and innate immunity.

## Materials and methods

### Mice

C57BL/6J mice (B6; CD45.2$^+$) and congenic B6.SJL-Ptprc$^a$ Pep3$^b$/BoyJ (CD45.1$^+$) mice were purchased from Charles River Laboratories. CD11c.DTR mice [18] were obtained from S. Jung (Weizmann Institute, Rehovot, Israel). Mice were held under specific-pathogen-free conditions at the central animal facility of the German Cancer Research Center, and experiments were according to institutional guidelines and regulations.

For generation of CD11c.DTR BAC transgenic mice, a fusion construct composed of the DNAs for the human DTR, ovalbumin fragment aa 140–386, and eGFP was generated and inserted at the start codon of the CD11c gene in the BAC RPCI-24-7812 using *Escherichia coli* DH10B [25]. The final construct was linearized using *Not*I and injected into the pronuclei of fertilized C57BL/6N mouse eggs. The resulting transgenic mouse line was designated CD11c.DOG (*D*TR–*O*VA–e*G*FP). All experiments were performed with heterozygous mice for the DOG construct. CD11c.DOG mice were CD45.2 homozygous. Genotyping was carried out by PCR from genomic DNA of tail biopsies using the following primers for OVA: AACCTGTGCAGATGATGTACCA and GCGATGTGCTTGATACAGAAGA. For systemic DC depletion, CD11c.DOG mice were injected i.p. with 8–64 ng/gbw DT (Sigma, in PBS) as indicated.

### Generation of mixed bone-marrow chimera mice

Mixed bone-marrow chimera mice were made by transferring $2 \times 10^6$ T-cell depleted donor bone-marrow cells of each CD11c.DOG $\times$ CD45.1/1 mice (DTR$^+$, CD45.1/1$^+$) and B6 or IL-15$^{-/-}$ (DTR$^-$, CD45.2/2$^+$) mice at a ratio of 50:50 into ten Gy-irradiated recipient B6 mice. Experiments were started 8–10 wk after reconstitution.

### *In vivo* NK-cell kill assay

NK cytotoxic activity was assayed as previously described [26]. Briefly, mice received 30 μg CpG ODN 1668 (MWG Biotech AG) i.p. for activation of NK cells. As target cells, splenocytes from naive B6 and Tapasin-deficient (Tpn$^{-/-}$) mice [27] were labelled with 0.2 or 2 μM CFSE (Molecular Probes/Invitrogen), and $10^8$ cells of each target populations were co-injected i.v. into the recipient mice. After 2 h, spleens were harvested, single-cell suspensions prepared and the proportions of differentially CFSE-labelled target cells assessed by flow cytometry.

### Adoptive transfer of NK cells

NK cells were purified from spleens of CD45.1/.1 congenic hosts using anti-DX-5 microbeads (Miltenyi Biotec) according to the manufacturer's instructions. Purity of NK cells (NK1.1$^+$CD3$^-$) was typically $>80\%$. DX-5$^+$ cells were labelled with 1 μM CFSE (Molecular Probes/Invitrogen). A total of $10^6$ cells was transferred i.v. into recipient mice rendered lymphopenic by sublethal irradiation (6 Gy, X-ray source, 0.701 Gy/min, 1 day before transfer of DX-5$^+$ cells).

### Flow cytometry

Biotinylated or fluorochrome-labelled antibodies were obtained from BD Biosciences or eBiosciences: CD45.1 (A20), CD45.2 (104), CD11c (HL3), I-A$^b$ (KH74), I-A/I-E (2G9 or M5/114.15.2), DX-5 (DX5), NK1.1 (PK136). Phycoerythrin- or APC-Cy7-labelled streptavidin was obtained from BD Biosciences. Propidium iodide was used as a viability dye. Labelled cells were measured on a FACScalibur or FACSCanto II (BD Biosciences) and analysed using FlowJo 6.4.7 software (Tree Star).

**Acknowledgements:** We thank G. Küblbeck, S. Schmidt and F. van der Hoeven for assistance in the generation of CD11c.DOG mice; C. Schmidt-Mbamunyo and M. Wühl for assistance in experimental procedures. A. Kissenpfennig and B. Malissen (Centre Immunologie de Marseille-Luminy) kindly provided a human DTR cDNA, and S. Jung (Weizmann Institute, Rehovot, Israel) generously provided the CD11c.DTR mice. This work was partially supported by EU projects NoE-MUGEN (LSHG-CT-2005-005203) and Cancer Immunotherapy (LSH-2004-2.2.0-5), by the German Ministry of Education and Research (NGFN-2 01GS0452), and by Tumorzentrum Heidelberg-Mannheim (D.100.27.963).

**Conflict of interest:** The authors declare no financial or commercial conflict of interest.

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*Abbreviations:* **BAC:** bacterial artificial chromosome · **DT:** diphtheria toxin · **DTR:** diphtheria toxin receptor · **gbw:** gram body weight

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**Supporting Information for this article is available at www.wiley-vch.de/contents/jc_2040/2008/38659_s.pdf**

Received: 30/6/2008
Accepted: 28/7/2008

 