Novel function for interleukin-7 in dendritic cell development

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Abstract

Interleukin-7 (IL-7) is crucial for the development of T and B lymphocytes from common lymphoid progenitors (CLP) and for the maintenance of mature T lymphocytes. Its *in vivo* role for dendritic cells (DC) has been poorly defined. Here we investigated whether IL-7 is important for the development or maintenance of different DC types. Bone marrow-derived DCs expressed the IL-7 receptor (IL-7R) and survived significantly longer in the presence of IL-7. Migratory DCs (migDC) isolated from lymph nodes also expressed IL-7R. Surprisingly, IL-7R was not required for their maintenance but indirectly for their development. Conventional DCs (cDC) and plasmacytoid DCs (pDC) resident in lymph nodes and spleen were IL-7R-negative. Using mixed bone marrow chimeras we observed an intrinsic requirement for IL-7R signals in their development. As the number of CLPs but not myeloid progenitors was reduced in the absence of IL-7 signals, we propose that a large fraction of cDCs and pDCs derives from CLPs and shares not only the lymphoid origin but also the IL-7 requirement with lymphocyte precursors.

Introduction

Dendritic cells (DCs) are antigen-presenting cells that are critical for inducing immunity as well as tolerance of T cells. Based on phenotype, localization and function, several CD11c+ DC subsets can be identified in murine secondary lymphoid organs (SLOs). Conventional DCs (cDCs) are resident within SLOs, have an immature phenotype (MHCIIint) and sample antigens locally. They can be divided into a CD8α+ and a CD8α− subpopulation that differ in immune function, cytokine expression and antigen presentation. In contrast, plasmacytoid DCs (pDCs, MHCIIlow) are poor antigen-presenting cells and produce type I interferon after stimulation by viral or bacterial infection. In skin-draining lymph nodes (LN) a third DC type often referred to as migratory DCs (migDCs, MHCIIhi) is found. These comprise dermal DCs and epidermal Langerhans cells, which capture antigens in the skin and migrate via the lymphatics to the draining LN to activate antigen-specific T cells.
The capacity of DCs to present self as well as foreign antigens in SLOs is limited due to their rapid turnover. Under steady-state conditions, cDCs in spleen and LN are almost completely replaced within 3-5 days, while pDC in spleen and migDCs in LN have an approximate half-life of 10 and 20 days, respectively. Only a few factors regulating the maintenance of lymphoid tissue DCs in vivo are known, including lymphotxin β-receptor (LTβR) and bcl-xL. However, the short life span of cDCs in resting SLOs suggests that they are continuously replaced by precursor cells immigrating into the tissue. Some of the replacement may also occur locally as indicated by the presence of proliferating DC precursors. The precursors of most DCs are thought to reside in the bone marrow (BM) and share early progenitors with other hematopoietic cell lineages. Hematopoietic stem cells (HSCs) decide first between a lymphoid versus myeloid cell fate by differentiating into either common lymphoid progenitors (CLP) or common myeloid progenitors (CMP). CLPs can give rise to NK, T and B cells, while CMPs can become macrophages, granulocytes, erythrocytes and megakaryocytes. In addition, both CLPs and CMPs have the potential to give rise to cDCs and pDCs in mice and men suggesting considerable plasticity in DC development. However, the relative contributions of CLPs and CMPs to the peripheral DC pool is only partially understood.

Over the last few years, several cytokines and transcription factors regulating DC development have been identified. The cytokine FMS-like tyrosine kinase 3 ligand (Flt3L), which binds to its receptor Flt3 and signals via the transcription factor Signal Transducers and Activators of Transcription (STAT) 3, is important for DC development in vitro and in vivo. Interestingly, adult Flt3L- and STAT3-deficient mice still develop 10-65% of splenic cDCs and pDCs indicating the involvement of other factors. A good candidate is STAT5 as mice lacking this factor show a 60% reduction in splenic cDC and pDC numbers. However, granulocyte-macrophage colony-stimulating factor (GM-CSF), which binds to the GM-CSF receptor and signals via STAT5, was shown to be dispensable for DC development in vivo. These findings suggest that an unidentified factor signaling through STAT5 contributes to DC development.
The IL-7 receptor (IL-7R) is one of the best-characterized receptors that signals through STAT5. IL-7 is its only ligand and was shown to be crucial for the development of αβ, γδ and NK T cells in the thymus as well as B cells in the BM (reviewed in 21,22). In addition, IL-7 expressed within SLOs provides survival signals for mature re-circulating αβ T cells.23 To date, the role of IL-7 for DCs is poorly understood. Spleens of IL-7Rα−/− mice were shown to have fewer pDCs and cDCs24, which could be secondary to the B cell deficiency.25 Another study based on IL-7Rα−/− BM chimeras suggested that DC development is not IL-7 dependent.26 We have reinvestigated the role of IL-7 for DC development and homeostasis using several in vivo models. Intriguingly, all of our data support the conclusion that IL-7 plays an important role in the development of cDCs and pDCs.

Methods

Mice and BM chimeras

C57BL/6 (B6) mice were from Janvier (Le Genest Saint Isle, France); CD45.1+ and CD45.2+ B6 mice were from Jackson. IL-7−/− mice27, IL-7Rα−/− mice28, IL-7tg mice29 and TSLP-R−/− mice30 have been previously described. For BM chimeras, CD45.1+/−/2+ recipients (F1 of CD45.1+ x CD45.2+) were lethally γ-irradiated (2x 450 rad in 6 h interval) and then injected i.v. with 10^7 total BM cells from CD45.1+ WT mice, CD45.2+ IL-7Rα−/− mice or a mixture of both (ratio 1:1). Chimeras were analyzed 16 weeks after reconstitution. Mice within each experiment were sex- and age-matched. All mice were maintained under pathogen-free conditions. All mouse experiments were approved by the Swiss Federal Veterinary Office.

Cell isolation

LN (axillary, brachial, inguinal) and spleen were dissected from CO2-euthanized mice. Capsules of LN were opened with 26-gauge needles and spleens cut into small pieces. Tissues were digested for 30 min at 37°C in RPMI 1640 medium (Invitrogen, Basel, Switzerland) containing Collagenase D (1 mg/ml; Roche, Rotkreuz, Switzerland), DNAse I (40 mg/ml; Roche) and 2.5 % (vol/vol) Fetal Calf Serum (FCS; Sigma-Aldrich, Buchs, Switzerland). Subsequently, EDTA
(Ethylenediaminetetraacetic acid) was added (5 mM final) and cells were gently pipetted, passed through a 40-μm mesh, washed twice and resuspended in RPMI medium containing 10% FCS. For cell isolation from ears, ears were split with forceps and incubated in PBS with 0.5% trypsin (Sigma) and 5 mM EDTA for 20 min at 37°C to separate dermal and epidermal sheets. To isolate DCs both sheets were digested for 2h with Collagenase D as described above. Cells were counted by excluding dead cells stained with Trypan blue dye. Stromal cells were isolated from LNs as described before.23

**BMDC generation and culture**

BMDCs were generated as described previously.31 Briefly, BM cells were obtained from femur and tibia of B6 mice by crushing bones with a mortar. After filtering through a 40 μm mesh, BM cells were cultured for 10 days in complete IMDM medium (Invitrogen; containing HEPES (10 mM), penicillin (50 IU/ml), streptomycin (50 μg/ml), β-mercaptoethanol (50 μM), 10% FCS and 10% (vol/vol) GM-CSF-containing culture supernatant (COS line donated by F. Tacchini-Cottier) in untreated 100 mm plastic dishes. Medium was changed on days 3, 6 and 8 of culture. BMDCs were harvested on day 10 by gentle pipetting. For survival assays, 0.1x10⁶ BMDCs per well were cultured (flat bottom 96-well cell culture plates) in complete RPMI 1640 medium supplemented with 10% (vol/vol) FCS. For some experiments, BMDCs were labeled with 20 μM CFSE (Molecular Probes, Invitrogen). For in vitro blocking experiments, antibodies (online supplemental table S3) were added to the culture in the indicated concentrations.

**Flow cytometry**

1.5x10⁶ cells were blocked with 2% (vol/vol) normal mouse serum (Sigma) for 20 min on ice and then stained with antibodies (see online supplemental table S1) in PBS containing 2% FCS, 2 mM EDTA and 0.1% (wt/vol) NaN₃ for 30 min on ice. Biotin-conjugated primary antibodies were detected with secondary reagents (see online supplemental table S2). Dead cells were excluded using 7AAD or DAPI (Molecular Probes, Invitrogen). Data were acquired on a FACSCanto
(Becton Dickinson, San Jose, CA) or a Cyan APD (Beckman-Coulter, Fullerton, CA) and analyzed with FlowJo software (TreeStar, Ashland, OR).

**BrdU labeling and detection**

BrdU (Bromodeoxyuridine, Sigma) administration to mice as well as DC surface-staining (using doubled antibody concentrations) and fixation were as described before, except that cells were incubated with 70 Kunitz U/ml DNAse I (Roche) for 45 min at 37°C and with FITC-conjugated α-BrdU mAb or an isotype matched control mAb (Becton Dickinson) for 60 min at room temperature.

**Immunofluorescence microscopy**

Staining and microscopy of cryosections were performed as previously described. For antibodies, see online supplemental table S4.

**RNA isolation and quantitative RT-PCR.**

RNA extraction, primers and quantitative realtime PCR from total LN, spleen and BM RNA were as previously described. Efficiency-corrected expression of *Il-7* was normalized by division of that expression with the geometric mean of expression of the ‘housekeeping’ genes encoding hypoxanthine guanine phosphoribosyl transferase (*Hprt1*) and TATA-binding protein (*Tbp)*.

**Statistical analysis**

Statistical significance was determined using an unpaired two-tailed Student’s t-test for unequal variances. p-values < 0.05 were considered statistically significant.

**Results**

**IL-7 mediates survival of BM-derived DCs in vitro**

IL-7 responsiveness is limited to cells expressing the IL-7R, a heterodimer composed of the IL-7Rα chain (CD127) and the common γ (γc) chain (CD132). We confirmed a previous report...
showing the expression of IL-7Rα on GM-CSF-matured BMDCs (Figure 1A). We also observed expression of γc chain on these cells suggesting that BMDCs can express a functional receptor for IL-7. As IL-7 is a potent survival factor for naïve T cells in vivo and in vitro, we tested whether IL-7 has a similar effect on BMDCs. In vitro, BMDCs survived significantly better in the presence of increasing concentrations of recombinant IL-7 protein (Figure 1B). As LN DCs are in close contact with IL-7 producing TRC after their arrival in the LN T zone, we cultured BMDCs on a layer of TRC-containing LN stromal cells or with supernatant derived from such LN stromal cell cultures. In both cases 60-80% of BMDCs survived for 24h, in contrast to only 40% survival in the absence of stromal cells or their supernatant (Figure 1B). This survival effect could be almost completely abolished by adding neutralizing antibodies to IL-7 or to IL-7Rα (Figure 1C). Around 60% of the BMDCs survived longer than 5 days in co-culture with LN stromal cells, which represents a 5-fold prolongation of the BMDC half-life compared to the no-stroma control (Figure 1D). Based on CFSE (Carboxyfluorescein succinimidyl ester) dilution experiments this survival was independent of DC proliferation (data not shown). Together, these results indicate that the IL-7R expressed on BMDCs is functional and promotes their survival.

**IL-7 receptor is expressed by steady-state migDCs but not cDCs or pDCs**

To test whether IL-7 plays a role for DC development and homeostasis in vivo, DC subsets in spleen and LN of naïve mice were first stained for the IL-7R complex. DC subsets were defined based on the expression levels of CD11c, MHCII, CD8α and PDCA-1 (Figure 2A and data not shown). CD11c+ MHCIIhi migDCs isolated from LNs expressed IL-7Rα at levels similar to CD8α+ T cells (Figure 2B). The direct ancestors of migDCs in the skin, namely dermal and epidermal DCs, also expressed the IL-7Rα chain (Figure 2C), consistent with data on human dermal DCs. In contrast, and similar to B cells, no IL-7Rα expression was detectable on CD8α− cDC subsets (CD11c+ MHCIIint) and pDCs (CD11c− PDCA-1+) (Figure 2B). The γc chain was expressed by all cell types analyzed, including all DC subsets (Figure 2D). In conclusion, migDCs but not cDCs or pDCs have the potential to respond to IL-7.
IL-7 overexpression increases cDC and pDC numbers in SLOs

On the one hand, the IL-7R expression on migDCs suggests that their steady-state numbers in LNs may be regulated by the amount of IL-7 produced by TRCs, similar to naïve T cells. On the other hand, IL-7R^- cDCs and pDCs may depend on IL-7 for their development but not their maintenance, in analogy to B cells. To test these hypothesis in vivo, DC numbers were assessed in wild-type (WT) mice and mice over-expressing IL-7 under the control of the MHCII-promoter. These IL-7 transgenic (tg) mice express 10-30 fold more IL-7 transcripts in BM, pLN and spleen than WT mice (Figure 3A). While a profound increase in T and B cell numbers has already been reported for SLOs in these mice, DCs have not been investigated in this setting. Analysis by flow cytometry revealed that IL-7 overexpression reduced migDC proportions in LNs in comparison to WT tissues (Figure 3B). Quantification of migDC numbers in LN revealed no difference between IL-7tg relative to WT tissues arguing against a major role of IL-7R in migDC development or maintenance (Figure 3C). In contrast, the proportion of cDCs and pDCs was unchanged (Figure 3B; not shown). Consequently, numbers of CD8α^+ and CD8α^- cDCs as well as pDCs were found to be strongly increased in LNs and spleen in an extent similar to IL-7-dependent lymphocytes. Since both cDCs and pDCs in SLOs do not express detectable IL-7R levels these results suggest a developmental effect of IL-7 overexpression on these DC subsets, similar to the effect of IL-7 for B lymphocytes. Alternatively, the IL-7 effect on steady-state DCs could be indirect. As expected, splenic granulocytes that develop and function independently of IL-7 were unaffected by overexpression of IL-7.

Reduced DC numbers in two mouse models deficient for IL-7 signaling

To further investigate a possible role of IL-7 for DCs, we analyzed LNs and spleen of IL-7^-/- mice. As previously described, LNs were partially absent in IL-7^-/- mice (data not shown) and often significantly smaller than LNs in WT mice (Figure 4A). Splenic white pulp cords were also much smaller (Figure 4A) consistent with the severely reduced T and B cell numbers reported for IL-7^-/- spleen. So far, DCs have not been investigated in IL-7^-/- mice. Histologically, no striking difference was observed in the localization of DCs in LN and spleen of IL-7^-/- compared to WT
mice (Figure 4A). However, their number per tissue section seemed to be reduced. Flow cytometric analysis of DCs in IL-7−/− LNs and spleen confirmed that all DC subsets were strongly decreased in numbers while splenic granulocytes were not affected (Figure 4B). Notably, the DC defect was stronger in LN than in spleen.

To confirm the above findings in a system where indirect effects due to abnormally developed SLOs can be excluded, lethally irradiated CD45.1/2+ WT recipients were reconstituted with either CD45.1+ WT BM or CD45.2+ IL-7-Rα−/− BM. 16 weeks after reconstitution with IL-7-Rα−/− BM, SLOs contained hardly any T and B lymphocytes but normal numbers of splenic granulocytes, as expected (Figure 4C). All four DC subsets did develop, replicating the findings of a previous study using IL-7Rα−/− BM to reconstitute sub-lethally irradiated mice. However, that study did not report an effect of IL-7Rα deficiency on DC numbers. We found that IL-7-Rα−/− BM poorly reconstituted the DC compartment in LN and spleen compared to WT BM, with the reduction in DC numbers ranging from 25% to 85% (Figure 4C). Similar to the IL-7−/− mice, the reduction in cDCs and pDCs in LNs was more pronounced than in the spleen. Contrary to the previous study, our results suggest that IL-7 is a critical regulator of steady-state DC numbers in SLOs.

**Intrinsic IL-7Rα requirement for cDC and pDC development**

To test whether DC development is directly regulated by IL-7 acting on IL-7R+ DC precursors or indirectly via other IL-7R expressing cells mixed bone marrow chimeras were generated by reconstitution of lethally irradiated CD45.1/2+ WT mice with an equal number of WT (CD45.1+) and IL-7Rα−/− (CD45.2+) BM cells. The allelic markers together with the competitive situation allow to determine whether DCs have a preference to derive from BM precursors of WT or IL-7Rα−/− donor origin (Figure 5A). In addition, it generates normal T and B cell compartments in SLOs as virtually all lymphocytes are derived from WT precursors (not shown). Interestingly, cDCs and pDCs found in LN and spleen showed a statistically significant preference to derive from WT rather than IL-7Rα−/− BM precursors (Figure 5B,C; see Supplementary Figure 1 for pDC gating).
As we did not detect IL-7R expression on cDCs and pDCs in SLOs, these results indicate an intrinsic requirement for IL-7 signals at the level of their precursors. In contrast, migDCs in the LN as well as their direct ancestors in the dermis were equally derived from WT and IL-7Rα−/− BM cells (Figure 5B). The IL-7 independent splenic granulocytes also developed equally well from both BM sources (Figure 5C). Given the reduction of migDCs in IL-7−/− mice, it suggests an indirect requirement of IL-7Rα signals in migDC development or accumulation.

To assess whether IL-7 signals influence the rate by which steady-state DCs are replaced with a proliferating precursor, we fed the mixed chimeras with the nucleotide-analogue BrdU over 3 days. The frequency of BrdU+cDCs and migDCs in SLOs was comparable for WT and IL-7Rα−/− donor cell populations (Supplementary Figure 2). This result indicates that IL-7 signals do not influence the rate of DC generation from the immediate DC precursor. Moreover, it strengthens our conclusion that the survival of IL-7R+ migDCs in LNs is not directly controlled by IL-7 signals.

Because the IL-7Rα chain can also pair with the thymic stromal lymphopoietin (TSLP) receptor α chain to form the TSLP-R30 the use of IL-7Rα−/− BM cells might also affect TSLP-R function. However, DC numbers were not reduced in SLOs of TSLP-Rα−/− mice (data not shown), confirming previously published results.30 Given that the IL-7Rα−/− BM chimeras faithfully replicated the DC defect seen in IL-7−/− mice we conclude that IL-7 but not TSLP is critical for the development of normal DC numbers in vivo.

Effect of IL-7 on DC precursors in the bone marrow

The various mouse models strongly suggest that not only precursors of lymphocytes but also of cDCs and pDCs depend on IL-7 for their development. To test this hypothesis, we analyzed the BM of IL-7 overexpressing and IL-7-deficient mice for hematopoietic precursors known to have DC potential in vivo, including CLP, CMP and common dendritic cell precursors (CDP). CDPs have only DC potential and give rise to both cDCs and pDCs in vitro and in vivo.17 Among these
three precursors only CLPs express IL-7Rα (Figure 6A; not shown) and could directly respond to IL-7 present in the BM.\textsuperscript{30} IL-7 overexpression doubled the total BM cell number (31.2 ± 2.5 x10^6 in WT vs. 62.2 ± 4.0 x10^6 in TG mice), probably due to increased B cell development.\textsuperscript{36} However, it did not alter the size of these three precursor populations presumably due to the known IL-7 mediated relocalization of hematopoietic precursors to the spleen (Figure 6B).\textsuperscript{37} In IL-7\textsuperscript{−/−} BM only the CLP population was strongly reduced in size (Figure 6C), confirming an earlier report.\textsuperscript{38} The Flt3\textsuperscript{+} and Flt3\textsuperscript{−} CLPs were equally affected (not shown). The IL-7 requirement was cell-intrinsic as in mixed chimeras CLP-enriched cells (Lin\textsuperscript{−} c-Kit\textsuperscript{int} Sca-1\textsuperscript{int}) showed a statistically significant tendency to derive from IL-7Rα-expressing BM cells (Figure 6D). While CDPs were not reduced in an IL-7 deficient background, in mixed chimeras they showed a strong trend to derive from IL-7Rα\textsuperscript{±} BM (Figure 6C, D). In contrast, the size of the myeloid progenitors, including common myeloid progenitors (CMPs), granulocyte-monocyte progenitors (GMPs) and megakaryocyte-erythrocyte progenitors (MEPs)\textsuperscript{39}, was affected neither by lack of IL-7 nor IL-7Rα (Figure 6C, D).

In conclusion, only the homeostasis of CLPs is IL-7 dependent and correlates with the reduced number of cDCs and pDCs observed in SLOs of IL-7\textsuperscript{−/−} mice.

**Discussion**

In this study we have identified IL-7 as an important factor controlling steady-state DC numbers *in vivo* by regulating DC development rather than maintenance. This developmental defect was due to an intrinsic requirement of IL-7R on precursors of cDCs and of pDCs and an indirect requirement for precursors of migDCs. In contrast, IL-7 had a pro-survival effect on mature BMDCs.

BMDCs expressed the IL-7Rα chain\textsuperscript{32} and addition of IL-7 triggered their survival *in vitro*. IL-7 producing LN stroma was efficient in augmenting BMDC survival to an extent similar to the one reported for naïve T cells.\textsuperscript{23} These data indicate that the IL-7-producing TRC found in the T zone of LN and spleen have the potential to regulate the survival time of IL-7R-expressing DCs.
Currently, we do not have evidence for this process to occur in vivo. However, these findings may have an implication for the clinical use of human monocyte-derived DCs, which were found to strongly increase IL-7Rα mRNA expression upon activation by various stimuli.\textsuperscript{40,41} Therefore the IL-7 responsiveness of DCs derived from human blood should be tested as it may improve their viability and therefore their efficiency in clinical use.

We found that migDCs express IL-7R at the cell surface and depend on IL-7 signals for normal steady-state numbers in SLOs. Therefore, IL-7 appeared as an attractive candidate regulating either migDC development or migDC maintenance or both, similar to T cells.\textsuperscript{21,22} However, the IL-7 independence of BrdU incorporation and numbers of migDCs in SLOs of IL-7Ra\textsuperscript{−/−} chimeras and IL-7tg mice argues against a role in their maintenance and for a role in their development. Accordingly, the effect on migDC development must be indirect via other IL-7R\textsuperscript{+} cells, such as lymphocytes. Mature T cells are the major IL-7Rα expressing cell type in SLOs. However, increased lymphocyte numbers in IL-7tg mice apparently did not increase migDC viability nor did TCRβδ\textsuperscript{−/−} mice show decreased migDC numbers (our unpublished observation). Therefore, our data are consistent with the notion that this indirect effect occurs at the migDC precursor stage possibly in the BM where other IL-7Rα\textsuperscript{+} cells, including B cell precursors, are present. As myeloid progenitors were not altered by the varying levels of IL-7R signals investigated, we favor the model that IL-7 indirectly affects a developmental stage between CMPs and migDCs. It remains an open question why steady-state migDCs express the IL-7R complex. IL-7 is constitutively expressed by keratinocytes and could increase the life span of skin DCs.\textsuperscript{42} Alternatively, IL-7R on skin DCs and migDCs could become functional during the immune response to improve or prolong antigen presentation.\textsuperscript{43} Interestingly, human monocyte-derived DCs can produce IL-7 themselves, especially after CD40 stimulation, which could augment both DC survival and T cell responses.\textsuperscript{44} Tools to delete IL-7Rα or STAT5 in a DC-specific and inducible manner will be helpful to address these unresolved issues.
In contrast to migDCs, cDCs and pDCs in SLOs did not express the IL-7R complex. Nevertheless, the homeostatic number of both cDCs and pDCs was closely linked to the level of IL-7 expression arguing for a role in development and not in maintenance. Further supporting this notion, mixed BM chimera experiments established a cell-intrinsic role for IL-7Rα in precursors of cDCs and pDCs. Previously, Takeuchi and Katz\textsuperscript{26} had proposed that DC development is independent of IL-7. While we confirmed that IL-7Rα\textsuperscript{−/−} BM cells can give rise to all DC subsets, we found a significant dependency of cDC and pDC numbers on IL-7 signals that was not described in the previous study. This discrepancy appears to be due to the different experimental design. Takeuchi and Katz used only one experimental system where they transferred IL-7Rα\textsuperscript{−/−} BM cells into sub-lethally irradiated WT mice that have lost highly proliferating DC precursors but not terminally differentiated DCs. Since steady-state DCs in SLOs have a very short half-life (3-20 days) the competitive situation was rapidly lost as indicated by 85-100% of DCs in SLOs being of donor origin 4 weeks after reconstitution. In addition, no absolute numbers of DCs were reported. In our study, we have used multiple \textit{in vivo} models all of which have indicated a role of IL-7 in controlling the development and thereby the steady state numbers of cDCs and pDCs. Our data support the concept that cDC and pDCs share a common precursor expressing IL-7Rα.

IL-7 has so far been a cytokine largely restricted to the lymphoid lineage. IL-7 is known to act as trophic factor for developing and mature αβ-T cells, as mechanistic signal for γ-locus recombination in developing γδ-T cells and as lineage commitment factor for developing B cells. In probably all lymphocyte precursors, IL-7 plays a role in the induction of RAG expression.\textsuperscript{21,22} So far, there was only limited evidence for a role of IL-7 in DC biology. \textit{In vitro} differentiation of CLPs or early thymic progenitors into DCs was shown to be more effective in the presence of IL-7 as it augmented cluster formation and DC generation.\textsuperscript{11,45,46} Cultures of splenocytes from IL-7Rα\textsuperscript{−/−} mice were observed to be less efficient in generating DCs than WT splenocytes presumably due to the reduced number of DCs found in IL-7Rα\textsuperscript{−/−} spleen.\textsuperscript{24,43} Finally, addition of IL-7 to fetal thymic organ cultures led to DC development.\textsuperscript{47} In our study, we report now \textit{in vivo} evidence supporting such a role in DC development. While some of the effects we observed on
steady-state numbers of cDCs and pDCs may be indirect, we obtained genetic evidence for a cell-intrinsic role of IL-7R in their development. So what function may IL-7 have for DC precursors? The strong reduction in CLP number within IL-7$^{-/-}$ BM$^{38}$ would be consistent with IL-7 acting as a trophic factor for CLPs eventually influencing the number of CLP progeny, including cDCs and pDCs. In future, this notion may be tested in IL-7$^{-/-}$ mice overexpressing the pro-survival factor Bcl-2. We do not believe that IL-7 is critical for RAG expression or IgH rearrangements in DC precursors as they were equally observed within CLP- and CMP-derived pDCs.$^1$ Intriguingly, cDCs found inside the LN and spleen do not express detectable IL-7R, thus possibly contributing to the poor survival of these cells in SLOs and leading to their rapid turnover.$^{3,4}$

IL-7 is constitutively produced by BM stromal cells and is likely to influence IL-7R$^+$ DC precursors within this environment.$^{22,23}$ To our knowledge, only more differentiated HSCs (LMPPs), CLPs and B cell progenitors have been reported to express IL-7R$\alpha$ in the BM.$^{17}$ As CLP numbers were dependent on both intrinsic IL7R$\alpha$$^{38}$ and normal levels of IL-7, they correlate best with the cDC and pDC numbers detected in SLOs. In contrast, we found the numbers of myeloid progenitors and CDPs to be IL-7 independent suggesting different cytokine regulation and possibly CLP-independent origin. Therefore, our data are consistent with a model in which some but not all cDCs and pDCs develop out of CLPs and may share this common precursor. This finding further strengthens the concept that these DCs arise from precursors shared with lymphocytes.$^{1,2}$ Interestingly, deficiency in this lymphoid pathway of cDC and pDC development cannot be compensated by the myeloid pathway. In contrast, migDCs belong to the myeloid lineage that does not depend on an IL-7 dependent precursor. This notion is supported by transplantation experiments demonstrating CMPs as major precursor population of Langerhans cells.$^2$

CMPs are commonly considered more important than CLPs in their contribution to the peripheral DC pool.$^{1,12}$ This notion is mainly based on the higher frequency of CMPs compared to CLPs. However, CLPs are typically found to be more efficient in generating DCs upon transfer in vivo.
The major surface receptor system promoting cDC and pDC development, Flt3, is expressed by a subset of both CLPs and CMPs. Although Flt3L−/− mice have normal CMP and a 9-fold reduction in CLP numbers Flt3L is thought to be important for both pathways.48 As a consequence, Flt3L−/− mice have a strong reduction in splenic cDC and pDC numbers ranging from 75-90%.14,20 In comparison, we observed a slightly weaker reduction in cDCs and pDCs of IL-7−/− spleen that was nevertheless in the order of 50%. In mixed BM chimera only 35% splenic cDC and 25% splenic pDC developed out of IL-7Rα−/− BM. The mixed bone marrow chimera approach may allow to estimate the relative contribution of the myeloid versus lymphoid pathway on cDC and pDC development if one assumes that IL7Rα−/− CLPs do not produce any DCs while CMPs in the IL-7Rα−/− environment develop normally into DCs. In that scenario, DCs derived from WT BM develop from either myeloid or lymphoid precursors while IL-7Rα−/− derived DCs develop exclusively from myeloid precursors. The difference in efficiency of WT relative to IL-7Rα−/− BM should then be due to DC precursors from the lymphoid pathway. Based on these assumptions we estimate the contribution of the lymphoid pathway to the cDC pool to be as high as the one from the myeloid pathway, and to be above 50% for the pDC pool. Therefore, the lymphoid contribution may be stronger than previously anticipated.1,12

STAT5 was recently reported to be critical for cDC and pDC development.18 Since IL-7 is known to induce phosphorylation of STAT5 in T cells21,22, we propose that the IL-7 receptor may be one of the critical signals upstream of STAT5 in cDC precursors. Interestingly, splenic DC numbers were slightly more reduced in the STAT5−/− fetal liver chimeras than in the IL-7Rα−/− BM chimeras. GM-CSF−/− mice were reported to have normal DC numbers in SLOs.19 It is therefore possible that in the absence of IL-7, GM-CSF or yet another factor could contribute to STAT5 phosphorylation and thereby to cDC development. In analogy to lymphocyte precursors, IL-7 signals provided by BM stromal cells to CLPs or other DC precursors may help in their survival and differentiation.21,22 Conceptually, a common regulator of lymphocyte and DC development may ensure the generation of the key partners responsible for mounting adaptive immune responses. A similar concept has been put forward for the induction of self-tolerance in the
thymus as thymocytes and DCs were shown to develop out of a common thymic lymphoid precursor.\textsuperscript{49} It is tempting to speculate that IL-7 and Flt3 cooperate in DC development, similar to their additive effect on B and T cell development.\textsuperscript{50}

In conclusion, we have shown here that the prototypic lymphocyte-specific cytokine IL-7 also plays a previously unappreciated role in DC development and thereby steady-state DC numbers in SLOs. Our data suggest that lymphoid-committed precursors are comparable to myeloid precursors in their contribution to the peripheral pool of cDCs and pDCs.

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**Author contributions**

T.K.V. performed most experiments, analyzed all data and wrote the manuscript; A.L. helped setting up BMDC survival assays; J.P. did the histological stainings; D.F. assisted with the BM chimera generation and provided mice and advice; S.A.L. designed and directed the study and wrote the manuscript. All authors critically reviewed the manuscript.

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Figure legends

**Figure 1. IL-7 supports BMDC survival in vitro.** (A) Flow cytometric analysis of day 10 WT BMDC (CD11c⁺ MHCII⁺) for IL-7Rα (middle, black line) and γc-chain expression (bottom, black line). As control, IL-7Rα staining is shown for IL-7Rα⁻/⁻ BMDC (middle, grey shading) or isotype-control antibody staining on WT BMDCs (bottom, grey shading). (B-D) Day 10 BMDCs were cultured in the absence of GM-CSF and their survival assessed by Trypan blue dye exclusion. No significant differences were observed between MHCII⁺ and MHCII⁻ CD11c⁺ BMDC (not shown) and were therefore pooled for the analysis. (B) BMDCs were cultured for 24h either alone (-), in the presence of increasing concentrations of recombinant IL-7 protein, on a layer of subconfluent LN stromal cells (ratio of 10 BMDC to one stromal cell) or with supernatant (SN) derived from a 3 day LN stromal cell culture. (C) Neutralizing antibodies to IL-7 (10 μg/ml) or IL-7Rα (20 μg/ml) were added to BMDC cultured for 24h with LN stromal cells. (D) Culture of BMDCs with or without LN stromal cells for 1, 2 or 5 days. P values (*, P < 0.05; **, P < 0.01; ***, P < 0.001) are relative to the same time point of the ‘no-stroma’ control. Data are representative of at least two experiments with three independent samples each.

**Figure 2. IL-7R is expressed on steady-state migDCs but not cDCs or pDCs.** DC subsets from pLN, spleen and skin were analyzed for IL-7Rα and γc-chain expression using flow cytometry. (A-B) Analysis of IL-7Rα expression on migDCs (CD11c⁺ MHCII⁺⁺), CD8α⁺ and CD8α⁻
cDCs (CD11c+ MHCIIint), pDCs (CD11c− PDCA-1+ CD45RA−), B cells (CD11c− MHCII+) and T cells (CD8α+ CD11c+) in pLN and spleen of WT (black) and IL-7Rx−/− (grey) mice. (C) Analysis of IL-7Rx expression on DCs (7-AAD− CD45+ MHCII+) isolated from the epidermis (upper panel) or dermis (lower panel) from WT (black) or IL-7Rx−/− (grey) mice. These cells were also CD11c+ (not shown). (D) Expression of γc-chain was assessed in WT mice using an antibody against γc-chain (black) or an isotype control antibody (grey). Data are representative of three experiments.

Figure 3. Increased cDC and pDC numbers in IL-7tg mice. (A) Il-7 mRNA was quantified by realtime PCR in pLN and spleen of adult WT and IL-7tg mice (n=3). Numbers indicate fold-increase in IL-7tg over WT mice. (B-D) Cells isolated from pLN or spleen of WT and IL-7tg mice were stained and analyzed by flow cytometry. (B) Representative dot blots are shown for cDCs (MHCIIint) and migDCs (MHCIIhi) in WT and IL-7tg tissues. Numbers indicate the percentage of DC subsets among hematopoietic cells found in total pLN (upper panel) or spleen (lower panel). (C) Bars depict the number of DC subtypes, T cells, B cells (all gated as in Figure 2) and granulocytes (Gr-1hi CD11bhi). Mean cell numbers in WT mice were defined as 100%. Relative cell numbers are shown for WT versus IL-7tg mice. *, P < 0.05; **, P < 0.01; ***, P < 0.001. Data in B-D are representative of a total of 3-9 mice per group.

Figure 4. Reduced numbers of all DC types in IL-7−/− mice and IL-7Rx−/− chimeras. (A) Immunofluorescence staining of adult pLN and spleen of WT and IL-7−/− mice. Anti-B220 and anti-CD3 stainings outline the B and T zones, respectively. Anti-laminin stainings visualize vessels, stromal cells, LN capsule and splenic marginal sinus. Anti-CD11c stainings on consecutive sections indicate the localization and density of DCs. Bar represents 100 μm. Data are representative of three independent experiments. (B) Cells isolated from pLN or spleen were stained and the indicated DC subtypes, T cells, B cells and granulocytes analyzed by flow cytometry (gating as in Figure 3). Mean cell numbers in WT mice were defined as 100%. Relative cell numbers per LN or spleen are shown for WT versus IL-7−/− mice. Data are representative of a total of 3-9 mice per group. (C) Irradiated CD45.1+ CD45.2+ B6 mice were reconstituted with
either CD45.2+ IL-7Rα−/− (n=3) or CD45.1+ WT BM (n=2) and analyzed after 16 weeks as described in (B) except for pDCs which were identified as small cells being CD11cint GR-1+ B220+ CD11b−. Only numbers of donor-derived cells are shown. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Figure 5. Intrinsic requirement for IL-7Rα in cDC and pDC development. Lethally irradiated CD45.1/.2+ mice were reconstituted with an equal mixture of WT BM (CD45.1+) and IL-7Rα−/− BM (CD45.2+) and analyzed by flow cytometry 16 weeks later. (A) Representative dot plot of CD45.1 and CD45.2 staining on CD11c+ cells from LN showing the gating strategy used to identify the donor origin of DCs. (B-C) Ratio of WT versus IL-7Rα−/− BM-derived cells is shown for DC subsets in pLN and skin (B) and in spleen (C). DC and granulocyte staining was as in Figure 4B, except pDCs which were identified as small CD11cint GR-1+ CD11b− cells. All pDCs expressed B220 (not shown). All epidermal DCs were radio-resistant and of host origin (not shown). Differences in reconstitution efficiencies between experiments were normalized by adjusting the ratio of IL-7Rα−/− and WT HSCs in the BM to 1. Bars represent the mean ± SD for three to five mice. Data are compiled from two experiments. Statistical significance is calculated relative to BM HSCs (*, P < 0.05; **, P < 0.01).

Figure 6. DC precursors in the bone marrow and their dependence on IL-7 signals. (A) Hematopoietic precursor cells in the bone marrow were analyzed by flow cytometry and identified as follows: HSCs (Lin− Sca-1+ c-Kit+), myeloid progenitors (MPs, Lin− Sca-1− c-Kit+; including CMPs, GMPs and MEPs), CLPs (Lin− Sca-1low c-Kitlow IL-7Rα+) and CDPs (Lin− c-Kitint M-CSFR+ Flt3+). (B-C) Precursor cells isolated from BM of IL-7tg (B) and IL-7Rα−/− mice (C) were analyzed by flow cytometry and cell numbers are shown as % of control mice (n=3). (D) Ratio of WT (CD45.1+) versus IL-7Rα−/− (CD45.2+) BM-derived cells in mixed BM chimeras (host: CD45.1/2+) is shown for BM HSCs, MPs, CDPs, CLP-enriched cells (Lin− Sca-1low c-Kitlow cells containing 50-70% CLPs) as well as BM granulocytes (Gran.) (n=3-5). Differences in reconstitution efficiencies
between experiments were normalized by adjusting the ratio of IL-7Rα−/− and WT HSCs in the BM to 1. Statistical significance is calculated relative to BM HSCs (*, P < 0.05; **, P < 0.01).
Figure 5

A. Flow cytometry analysis of CD45.2 (IL-7Rα−) and CD45.1 (WT) cells in total LN DCs.

B. Bar graph showing the ratio WT/IL-7Rα− for CD8α+ cDCs, CD8α− cDCs, pDCs, migDCs, dermal DCs, and granulocytes in pLN, skin, and spleen. Significant differences are indicated by * and **.

C. Similar bar graph as in B, comparing different cell populations in various tissues.
Novel function for interleukin-7 in dendritic cell development

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