HHS Public Access

Author manuscript

Nat Commun. Author manuscript; available in PMC 2012 December 21.

Published in final edited form as:

Nat Commun. 2012 ; 3: 1223. doi:10.1038/ncomms2223.

Identification and characterization of polyclonal αβ T cells with dendritic cell properties

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Abstract

An efficient immune response requires coordination between innate and adaptive immunity, which act through cells different in origin and function. Here we report the identification of thymus-derived αβ TCR$^+$ cells that express CD11c and MHC class II, and require FLT3L for development (T_{DC}). T_{DC} express genes heretofore found uniquely in T cells or DC, as well as a distinctive signature of cytotoxicity-related genes. Unlike other innate T cell subsets, T_{DC} have a polyclonal TCR repertoire andrespond to cognate antigens. However, they differ from conventional T cells in that they do not require help from antigen-presenting cells, respond to TLR-mediated stimulation by producing IL-12 and process and present antigen. The physiologic relevance of T_{DC}, found in mice and humans, is still under investigation, but the fact that they combine key features of T and DC cells suggests that they provide a bridge between the innate and adaptive immune systems.

Introduction

An efficient immune response requires coordination between innate and adaptive immunity[1]. In particular, crosstalk between dendritic cells (DC) and T cells is key in the initiation of adaptive immunity[2], and both of those cell types are thought to have clearly distinct roles during this process. DC, the most efficient antigen-presenting cells, process and present pathogen-associated antigens in the form of peptides loaded on MHC molecules. The course of a particular adaptive immune response is shaped by the maturation and activation status of DC, with immature DC leading to tolerance and mature DC to efficient immune responses. One way DC are induced to mature is ligation of pattern-recognition receptors (PRRs) by specific microbial-associated patterns[3], which results in upregulation of costimulatory molecules and MHC II, as well as production of pro-inflammatory cytokines such as IL-12 and TNFα. Upon maturation, DC migrate to T cell areas in the peripheral lymphoid organs, where they present antigen loaded on MHC II molecules to CD4$^+$ T cells. Some DC are also very efficient in cross-presentation of viral or endogenous peptides on

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Author Contributions: M.K. and I.M. performed experiments, and M.K., I.M., and J.D.A conceived the research, designed experiments, analyzed results, and wrote the paper.

Author Information: The authors declare no competing financial interests.

MHC I molecules to $CD8^+$ T cells. Recognition of MHC-complexes by the T cell receptor (TCR) combined with costimulation provided by mature DC results in a complete and effective adaptive T-cell response[2].

Although both arise from bone marrow progenitors, the developmental paths of DC and T cells diverge early and are thought to be as distinct as their functions. Conventional DC (cDC) originate from a common DC progenitor in the bone marrow and migrate to peripheral lymphoid organs[4]. Transcription factors such as PU.1, Ikaros, IRF8, RelB, and Batf 3 have been implicated in DC development, but due to their pleiotropic role and the high heterogeneity of DC subsets none of these can be used to exclusively define the DC lineage[5,6]. Two recent papers reported that the transcription factor Zbtb46 is expressed by cDC throughout their differentiation, and is a specific marker for cDC among immune cells[7,8]. Although no master regulator of DC lineage commitment has been described, interactions of FLT3 with its ligand (FLT3L) are necessary for DC development and homeostasis, because FLT3L-deficient mice lack DC in peripheral lymphoid organs[9].

In contrast to DC, T cell commitment occurs in the thymus, where T cell precursors undergo a multi-step process that leads to the generation of mature $CD4^+CD8^-$ and $CD4^-CD8^+$ T cells[10,11]. The most immature thymocytes are $CD4^-CD8^-$ (double negative, or DN) and can be separated into four different populations (DN1-4) based on expression of CD44 and CD25. DN1-DN2 thymocytes retain the plasticity to give rise to some myeloid cell types, including NK cells and thymic DC[11–14]. Commitment to the T cell lineage, and the subsequent recombination of the TCRβ locus and pre-TCR expression, takes place at the DN3 stage ($CD25^+CD44^-$), is Notch-dependent, and subsequent to the silencing of a number of transcription factors important for myeloid development, most notably PU.1[14].

Although innate and adaptive immune systems have been thought to act through different cells and mechanisms, recent studies provide several examples in which these two arms of the immune system appear to overlap. For example, some thymus-selected T cells, such as Natural Killer T cells[15] and most γδ T cells[16] are considered innate because of their limited T cell receptor (TCR) repertoire and prompt responses to non-peptide antigens. There is also evidence that some T cells can express low but detectable levels of Toll-like receptors (TLR)[17], which are normally involved in maturation and activation of DC and other innate immune cells[3]. However, the outcome of TLR triggering in T cells is different from that in innate immune cells, being confined to increased survival and costimulation[17,18].

These observations prompted us to ask if there exist cells that truly combine the molecular and functional characteristics of innate and adaptive immunity. We have identified of a novel population of thymus-derived cells that, like DC, require FLT3L for development and exhibit surface markers and functions of both DC and T cells (T_{DC}).Molecular profiling revealed that T_{DC} express genes characteristic of DC, T cells, and cytotoxic cells. T_{DC} expressed polyclonal αβ TCRs and responded to antigen, but unlike conventional T cells did not require help from antigen-presenting cells. Strikingly, T_{DC} responded to TLR-mediated stimulation by producing IL-12, and were able to process and present antigen to MHC II-restricted T cells.

Results

Identification of cells expressing DC and T cell markers

Conventional murine DC express high levels of CD11c and MHC II. Interestingly, we found that around 7% of splenic DC expressed αβ TCR, a distinguishing characteristic of conventional αβ T cells (Fig. 1a). These cells comprised 0.04% of spleen and 0.06% of lymph node cells (Fig. 1b), and because they expressed T cell and DC, but not other lineage markers (Fig. 1c), were termed T_{DC}. T_{DC} were positive for T cell markers such as Thy-1, CD27, and either CD4 or CD8β at the same ratio as conventional T cells (Fig. 1d and 1e). T_{DC} had a polyclonal Vβ repertoire comparable to conventional αβ T cells (Fig. 1f), in marked contrast to previously described semi-invariant innate T cell subsets[15]. Because CD11c can be expressed on activated T cells in some infectious settings[19–21], we asked if T_{DC} might represent a subset of activated T cells. T_{DC} displayed neither signs of recent activation ($CD69^{-}CD25^{-}IL\text{-}7R^{hi}$) nor showed an increase in T cell memory markers CD122 or CXCR3 (Fig. 1g). The homing molecules CCR7 and CD62L were expressed similarly in T cells and T_{DC}, but not in DC. In fact, expression levels of all of the tested T cell markers on T_{DC} mirrored the levels on T cells. One activation/memory T cell marker, CD44, was expressed on T_{DC}, but was expressed equally on DC, and thus in this case cannot be considered a T cell marker. Staining for DC markers revealed that T_{DC} expressed CD11c, MHC II, CD11b, F4/80, and FcRγ at intermediate levels with respect to conventional DC (Fig. 1h and Supplementary Fig. 1a) while non-DC-specific markers such as MHC I and CD45 were expressed at similar levels (Supplementary Fig. 1a). To test whether T_{DC} are T cells that acquired DC markers through intercellular transfer[22], cells from a reporter mouse that reveals transcriptional regulation of CD11c were examined. Just as with cell surface CD11c (Fig. 1h), T_{DC} EGFP expression driven by the CD11c promoter was at levels intermediate between T and DC (Fig. 2a and Supplementary Fig. 1b). Furthermore, highly purified (Supplementary Fig. 1c) T_{DC} expressed *ciita*, which regulates transcription of MHC II and is not expressed by murine T cells[23] (Fig. 2b). In the obverse approach, a GFP reporter driven by the Thpok regulatory elements, a transcription factor active in CD4 T cells[24], was expressed in $CD4^{+}$ but not $CD8^{+}$ T_{DC} (Fig. 2c). Therefore, expression of cell surface T and DC markers in T_{DC} is a reflection of their transcriptional regulation.

FLT3L is a hematopoietic growth factor often used to expand DC in vivo[25]. To test if T_{DC} resemble DC in this regard, after assessing that T_{DC} express FLT3 (Fig. 2d, left), C57BL/6 (B6) mice were injected with B16-FLT3L melanoma cells. As described for conventional DC[25,26], T_{DC} downregulated FLT3 (Fig. 2d, right) and expanded in response to FLT3L, resulting in a 5–6 fold increase in frequency (Fig. 2e). FLT3L-expanded T_{DC} had a cell surface phenotype identical to T_{DC} in unmanipulated mice (data not shown), and were used in experiments when indicated. We considered the possibility that T_{DC} represented rare T cell/DC doublets that escaped doublet-discrimination detection (Supplementary Fig. 1d). Arguing against this was that the levels of the T cell markers on T_{DC} were indistinguishable from conventional αβ T cells whereas DC markers were intermediate between T and DC. Moreover, a radiation bone-marrow chimera showed that each newly generated T_{DC} displayed only one of the two congenic markers, never both, indicating that they are not T/DC conjugates (Supplementary Fig. 1e). Perhaps the best evidence came from imaging

highly purified T_{DC} by confocal microscopy (Fig. 2f). Whereas T cells were $TCR^+CD11c^-MHC\ II^-$ and DC were $TCR^-CD11c^+MHC\ II^+$, each T_{DC} bore all three markers. T_{DC} size and morphology was similar to T cells, not DC, suggesting that they are a novel subset of T cells with innate cell characteristics.

T_{DC} have a unique gene signature

cDNA microarray analyses were performed to provide a non-biased assessment of T_{DC} gene expression, and revealed that T_{DC} have a clearly distinct genetic profile (Fig. 3a). T_{DC} expressed many genes encoding proteins normally found in T cells (e.g. Lck, CD3 subunits, and IL-7Rα) or DC (CD83, MHC II, Flt3, and Zbtb46), but not both (Table 1 and 2). Many of the cell surface proteins identified by microarray were among those detected on T_{DC} in Fig. 1. In addition, T_{DC} expressed *itgal* (encoding CD11a, the alpha chain of the integrin LFA-1, which is upregulated in activated T cells) at levels comparable to those of DC and unstimulated T cells (data not shown). PU-1 (encoded by *sfpi1*) is a myeloid lineage-related transcription factor[5], and Zbtb46 has recently been shown to be expressed specifically in DC[7,8]. Their expression in T_{DC} and DC but not T cells was validated by RT-PCR (Fig. 3b). Interestingly, some T_{DC} associated genes were expressed at much higher levels than in T cells or DC. Among these were genes encoding granzymes A and B and eomesodermin, proteins normally expressed in cytotoxic cells, such as NK and effector $CD8^+$ T cells. The relatively high expression of these genes in T_{DC} was confirmed by RT-PCR, which revealed that *gzma* was detectable only in T_{DC}, whereas *gzmb* and *eomes* were expressed in T cells but at lower levels than in T_{DC} (Fig. 3b). Similar results were obtained with RT-PCR for *prf1*, which encodes cytotoxic molecule perforin. Intracellular staining revealed that ~20% of $CD8^+$ T_{DC} expressed granzyme B in the absence of stimulation, whereas although a small amount of mRNA was found by RT-PCR (Fig. 3b), the protein was undetectable in T cells (Fig. 3c). Therefore, in addition to sharing characteristics with T cells and DC, T_{DC} exhibited a distinct molecular profile exemplified by cytotoxic gene expression. It is notable that T_{DC} express a cytotoxic signature without signs of activation (Fig. 1g).

T_{DC} require a thymus and positive selection for development

Conventional αβ T cells require the thymus for development. Consistent with a similar requirement for T_{DC}, bone marrow cell cultures supplemented with GM-CSF or FLT3L did not give rise to T_{DC} (data not shown). In the thymus, a small percentage of cells expressing CD11c and MHC II was found within both the $CD3^{int}$ and $CD3^{hi}$ subsets (Fig. 4a). A requirement for T_{DC} development in the thymus was confirmed by the observation that T_{DC} were not detected in the spleens of athymic nude mice (Fig. 4b). To determine if T_{DC}, like αβ T cells, undergo antigen-specific selection in the thymus, mice expressing αβ TCR transgenes (Tg) on either positively-selecting or non-selecting MHC haplotypes were studied. $Rag2^{-/-}$AND TCR Tg thymocytes are positively selected on $H\text{-}2^b$ and $H\text{-}2^k$, but not the $H\text{-}2^q$ haplotype[27,28]. T and T_{DC} were present in mice with positively selecting haplotypes but not in mice of the non-selecting MHC, strongly suggesting that T_{DC} require the same thymic positive selection signals as conventional T cells (Figure 4c). Additional evidence that T_{DC} undergo positive selection came from studies of T_{DC} Thpok expression, a transcription factor essential for CD4 expression in mature T cells. As seen in conventional T cells[24], $Thpok^{-/-}$ mice lacked $CD4^+$ T_{DC}, and $CD8^+$ T_{DC} frequency was increased (Fig.

4d). To formally demonstrate that CD4 lineage-selectedT_{DC} were not lost but redirected to the CD8 lineage, a GFP-reporter that monitors Thpok promoter activity was used in Thpok-deficient mice[24] to allow detection of CD4 lineage-selected T_{DC} ($CD8^+GFP^+$) within the $CD8^+$ T_{DC} population (Fig. 4e). Together, these findings demonstrate that T_{DC} are similar to conventional T cells in their origin and development.

T_{DC} have T cell functional properties

The ability of T_{DC} to respond to TCR-mediated stimulation was assessed by stimulating splenocytes with anti-CD3. After stimulation T_{DC} displayed high levels of the activation marker CD69 (Fig. 5a). TCR-mediated T cell proliferation requires stimulation via costimulatory receptors whose ligands are provided by antigen-presenting cells (APC) such as DC[29]. Furthermore, stimulation with soluble anti-CD3 antibodies requires their immobilization by APC Fc receptors. Because T_{DC} exhibit DC characteristics and expressed CD80/CD86 (Supplementary Fig. 2a), we hypothesized that they might be able to provide these necessary functions for themselves. To test this, highly purified T cells or T_{DC} were stimulated with soluble anti-CD3. In contrast to T cells alone, T_{DC} expanded, and to a greater extent than T cells cocultured with DC (Fig. 5b), indicating that they do not need help from conventional APC. Interestingly, whereas T_{DC} activated via the TCR for up to 20 hours retained their DC markers (data not shown), they lost them after proliferating (Supplementary Fig. 2b). To address the possibility that this might be due to expansion of a very small number of contaminating conventional T cells, we crossed Rosa26-YFP mice, which express fluorescent YFP only if a stop codon between the Rosa26 promoter and YFP is excised by Cre[30], with CD11c-Cre-expressing animals. YFP expression was observed in the majority of DC and T_{DC}, and YFP^{bright} cells were isolated by sorting (Supplementary Fig. 2c) and stimulated with soluble anti-CD3 (Fig. 5c). T_{DC} expanded in culture and lost cell surface CD11c but maintained YFP expression. Importantly, YFP was not acquired by conventional T cells expanded in the presence of DC. Thus, T_{DC} expand in response to TCR-mediated stimulation. The question of whether T_{DC} respond to cognate antigen was addressed with αβ TCR Tg mice, all of which that we examined had T_{DC} (Supplementary Fig. 2d). T_{DC} sorted from OTII-Tg mice and stimulated with cognate antigen (chicken ovalbumin peptide 323-329) proliferated in the absence of any additional APC, whereas conventional T cells did not (Fig. 5d). Thus, T_{DC} have a functional TCR and are self-sufficient for antigen presentation.

To assess T_{DC} responses in vivo, equal numbers of LCMV gp33-41-specific P14 Tg T_{DC} and conventional T cells were adoptively transferred into wild-type hosts, which were then infected with LCMV. T_{DC} and T cell expansion was similar at day 8, the peak of the response (Fig. 5e). Moreover, comparable to the transferred T cells, all T_{DC} had an activated phenotype: $CD62L^{lo}$, $KLRG1^{hi}$, and $CXCR3^{hi}$ (Fig. 5f). Interestingly, T_{DC} lost expression of MHC II, acquiring a conventional T cell phenotype after LCMV-mediated expansion, as had been seen after proliferation in vitro. Analysis two months after LCMV infection, when numbers of both T and T_{DC} are at steady-state levels (Fig. 5g, left), found gp33 $tetramer^+$, and therefore antigen-experienced, T_{DC} that presumably had re-acquired MHC II (Fig. 5g, right). Notably, a relatively high percentage of T_{DC} were gp33-tetramer-positive, indicating that memory T_{DC} persist in vivo like conventional memory T cells.

T_{DC} respond to TLR agonists in a DC-like fashion

The innate characteristics of T_{DC} were explored. Unlike T cells, DC require signaling via FLT3 for development and maintenance[9]. Notably, the fraction of T_{DC} was markedly reduced in mice lacking the ligand for FLT3, FLT3L, to an extent similar to DC (Fig. 6a). To test the ability of T_{DC} to respond like innate immune cells, total splenocytes were stimulated with LPS (Fig. 6b) or other TLR agonists (Supplementary Fig. 3a). T_{DC} upregulated costimulatory molecules in a manner remarkably similar to DC (Fig. 6b), whereas no upregulation was observed for conventional T cells (data not shown). In addition, T_{DC} stimulated with TLR agonists produced IL-12, a cytokine produced by DC and important for Th1 polarization (Supplementary Fig. 3b). IL-12 production by T_{DC} was even more notable when splenocytes were primed with recombinant IFN-γ (rIFN-γ) followed by LPS stimulation (Fig. 6c and d). A prominent characteristic of DC is their ability to process and present antigens on MHC II. Purified T_{DC} or DC were co-cultured with OTII Tg T cells in the presence of whole ovalbumin protein. T_{DC} induced antigen-specific T cell proliferation similar to DC (Fig. 6e), indicating that T_{DC} can process as well as present antigen on MHC II. Thus, T_{DC} have functional properties characteristic of bona fide innate cells.

To determine if T_{DC} exist in humans, peripheral blood lymphocytes (PBL) from uninfected donors were analyzed. Approximately 0.2% of $CD3^{+}TCR\alpha\beta^{+}$ cells were positive for CD11c and HLA-DR (MHC II), but unlike murine T_{DC} the levels of these molecules were similar to those on conventional DC (Fig. 6f and Supplementary Fig. 3c). Human T_{DC} expressed T cell markers at the same levels as conventional T cells, and like their murine counterparts, were single positive for CD4 or CD8 (Supplementary Fig. 3d). Because activated human $CD4^{+}$T cells expressing MHC II have been described[31], we asked if human T_{DC} behave like innate immune cells. Human T_{DC} stimulated with LPS produced IL-12 (Fig. 6g). Treatment with recombinant IFN-γ prior to LPS stimulation increased IL-12 production by T_{DC}, as it did for DC.

Discussion

Here we report the identification of T_{DC}, a subset of cells characterized by the coexistence of features of conventional polyclonal T cells and DC. Similar to another rare but notable DC subset, plasmacytoid DC (pDC), T_{DC} have lymphocyte-like rather than DC morphology and express intermediate levels of CD11c and MHC II[32]. The fact that T_{DC} recombine and express a functional TCR, but also have the ability to respond to TLR stimuli in a rapid, DC-like fashion places T_{DC} in the heterogeneous category of “innate T cells”. However, T_{DC} substantially differ from other innate T cells primarily due to antigen-specific selection processes indistinguishable from classical αβ cells and the ability to mount a full-scale proliferative response to cognate antigens, both of which are essential in adaptive immunity. Moreover, they are only dull for CD122, CD69 and CD25, markers constitutively expressed by NKT cells, and do not express NK lineage markers such as NK1.1[16,33,34].

It is generally thought that conventional DC and T cells originate from different precursors and pursue unrelated developmental programs[4,11]. The identification of T_{DC}, which express lineage-specific markers of both DC (PU.1 and Zbtb46) and T cells (TCR αβ) suggests that

the lymphoid and myeloid differentiation pathways are not mutually exclusive, as previously thought. Whereas DN2 thymocytes, prior to T cell commitment, retain the potential to generate both myeloid and NK progeny[14], a mature cell seemingly committed to both T and DC lineages, with the capacity to respond in either a T- or DC-like fashion, has not been described. If anything, myeloid and T cell commitment are generally thought to be mutually exclusive. For example, forced expression of PU.1 at the DN3 stage inhibits T cell development, strongly arguing for its role in terminating T cell lineage commitment[35]. It is noteworthy, however, that signaling through Notch, initially thought to exclusively promote T cell lineage commitment, can also promote the development of $Thy1^+$ DC in bone marrow cultures[36] and the differentiation of some DC subsets in the periphery[37]. Such findings could perhaps explain how some cell types might escape what was thought to be a rule for T cell lineage commitment.

cDNA microarray analysis revealed that T_{DC} expressed a number of genes that were not found in DC or T cells. This distinct genetic profile identifies them as a separate cell type and raises the possibility that T_{DC} may have functions other than those of these two cell types. In particular, T_{DC} express granzymes and perforin at very high levels in the absence of signs of activation, which to our knowledge makes them the first naive polyclonal T cells with cytotoxic features to have been described thus far. Notably, only $T_{DC,}$ and not T cells or DC from naive mice, expressed Granzyme A, a protease with unique properties such as being able to activate alternative death pathways[38,39]. A recent paper reports that a subset of murine immature DC deletes cognate $CD8^+$ T cells through a mechanism mediated by perforin and Granzyme A[40]. This raises the speculation that T_{DC} might as well use their cytotoxic machinery to control an exacerbated immune response.

The physiological role of T_{DC} is currently being investigated by genetic approaches. Based on the fact that T_{DC} combine features and functions of T cells and DC, it is tempting to speculate that such duality might render T_{DC} particularly responsive to infectious organisms, because they can bridge innate and adaptive traits. The contribution of T_{DC} would be masked because, upon TCR-mediated proliferation, T_{DC} transiently lose DC markers, becoming indistinguishable from conventional T cells. However, as shown in responses to LCMV, antigen-experienced T_{DC} re-acquire DC markers over time, suggesting long-term stability of their “mixed” lineage. Infectious diseases are not the only context where T_{DC} might play a role. To our knowledge, T_{DC} are the first instance of an αβ T cell that produces IL-12. Several reports show that tumor-specific T cells engineered to produce IL-12 are very effective in eliciting regression of, or even eradicating, melanomas[41–43]. One could envision that tumor-specific T_{DC} might be effective in this setting because they produce IL-12 upon stimulation, similar to innate APC. Thus T_{DC}, present in mice and humans, comprise a new and intriguing cell subset poised at the intersection of innate and adaptive immunity.

Methods

Mice

C57BL/6 (B6) and athymic nude mice were obtained from the NCI-Frederick Animal Facility. FLT3L-deficient mice were generated by Immunex as described[16] and purchased from Taconic. CD11c-Cre, CD11c-Cre-EGFP, $ROSA26^{flox}STOP^{flox}YFP$, OTII TCR Tg,

AND TCR Tg, and B6 mice bearing CD45.1 or Thy-1.1 congenic markers were obtained from The Jackson Laboratory. Rosa26-CD11c-cre-YFP mice were obtained by breeding ROSA26^{flox}STOP^{flox}YFP to CD11c-Cre mice. Thpok-GFP, Thpok$^{-/-}$, and P14 TCR Tg in B6 background mice were a kind gift of Dr. Remy Bosselut (NCI). The Animal Care and Use Committee of the National Cancer Institute approved all animal experimental procedures.

Cells and reagents

Purified or fluorochrome-conjugated antibodies to murine and human cell surface markers and cytokines were purchased from BD Biosciences or eBioscience. LCMV Armstrong strain was a gift from Dr. Rafi Ahmed (Emory University School of Medicine). The H-2D^b/LCMV glycoprotein (gp)33-41 (KAVYNFATC) tetramer labeled with allophycocyanin was obtained from the NIH Tetramer Core Facility at Emory University.

Lipopolysaccharide (LPS), brefeldin A, and ovalbumin were purchased from Sigma Aldrich, PolyI:C and Pam_3CSK_4 from Invivogen, OVA 323-339 peptide from Genscript, CFSE from Invitrogen, and recombinant IL-2 and IFN-γ from eBioscience. B16-FLT3L murine melanoma cells were a gift from Ulrich H. von Andrian (Harvard Medical School).

B16-FLT3L-induced cell expansion in vivo

B16-FLT3L cells were cultured in DMEM containing 10% fetal calf serum and antibiotics. Mice were injected subcutaneously with 10^6 B16-FLT3L cells and lymphoid cells were analyzed 10–13 days later.

Cell preparation

Thymus, spleen, and lymph nodes were harvested and total cell suspensions made by gently mashing the debris through 40 μM nylon mesh (BD Biosciences). Viable cells were counted by Trypan Blue exclusion and the distribution of lymphoid populations in each organ was determined by cell surface staining and flow cytometry (LSRII, BD Biosciences). In some experiments, the indicated subsets underwent two or three rounds of sequential sorting to a purity of ≥ 99% (FACSAria I Cell Sorter from BD Biosciences). PBMC from healthy donors were isolated from buffy coats of healthy donors using a Ficoll gradient (Ficoll-Paque PLUS from GE Healthcare).

Confocal Microscopy

Splenocytes were stained with antibodies to CD11c, MHC II, and TCRβ (H57), sorted to > 99% purity and analyzed by confocal microscopy. Confocal images were sequentially acquired with Zeiss ZEN 2009 software on a Zeiss LSM 710 NLO Confocal system (Carl Zeiss Inc, Thornwood, NY) with a Zeiss Observer Z1 inverted microscope and diode laser tuned to 405 nm, a 25 mW Argon visible laser tuned to 488 nm and a HeNe laser tuned to 633 nm. A 63x Plan-Apochromat 1.4 NA oil immersion objective was used and digital images were 512 × 512 pixels with 0.264 mm pixel size. Emission signals after sequential excitation of eFluor450, FITC, and APC by the 405 nm, 488 nm or 633 nm laser lines were collected with a BP 419-485 nm filter, BP 495 -534 nm filter, and BP 650-710 nm filter, respectively, using individual photomultipliers.

Cell culture and stimulation

Cultures were performed with RPMI 1640 (Gibco) supplemented with 10% heat-inactivated fetal calf serum, glutamine, β-mercaptoethanol, and antibiotics. Murine splenocytes or human PBMC were stimulated with TLR agonists or with soluble anti-CD3ε (2C11) at the indicated concentrations. Activation markers were detected by surface staining 18–24 hr later. In some experiments, brefeldin A (Sigma-Aldrich) was added at a concentration of 10 μg/ml two hr after stimulation and after another 16–24 hr cells were fixed and permeabilized (BD Cytofix/Cytoperm and Perm/Wash Buffer kit) and stained for IL-12. In some proliferation experiments, double-sorted cells were stimulated with soluble anti-CD3 (1 μg/ml) and at day 3 the cultures were supplemented with IL-2 (20 U/ml) for 5 more days. When indicated, sorted cells were labeled with CFSE (0.5 μM) and stimulated with OVA peptide 323-339 or whole protein at the indicated concentrations.

Adoptive transfers and LCMV infection

P14 TCR Tg T cells from CD45.1$^+$ mice or P14 TCR Tg T_{DC} from Thy1.1$^+$ mice were sorted, mixed 1:1 (5000 cells total), and injected i.v. into B6 recipients. The day after adoptive transfer, mice were infected i.p. with 2×10^5 plaque-forming units of LCMV Armstrong strain, and spleen and lymph node cells were analyzed 8 days later. In other experiments, endogenous responses to LCMV were determined by infecting B6 mice with LCMV Armstrong strain and analyzed 2 months later.

Microarray analysis and RT-PCR

Total RNA was isolated with the RNeasy Micro kit (Qiagen) from 1000–4000 cells that were double or triple-sorted from B6 splenocytes. Microarray gene expression was performed by the Laboratory of Molecular Technology (NCI), with Affymetrix 430 2.0 GeneChip technology after RNA was amplified with a NuGEN WT-Ovation kit (Nugen). Microarray data analysis was performed with Partek Genomics Suite 6.6 (Saint Louis, MO). 1-way ANOVA was applied to 3 samples with paired comparisons between samples (T_{DC} vs. T, T_{DC} vs. DC and DC vs. T). For *ciita* detection by RT-PCR, cDNA was synthesized using SuperScriptTM II RT kit (Invitrogen) and RT-PCR was performed with *ciita* primers (5′-CCAAAGGATGTGGAAGACCT-3′ and 5′-AGCTTCTTAAGGTCCCGGAT-3′) and *HPRT* primers (5′-AAACAATGCAAACTTTGCTTTCC-3′ and 5′-TCCTTTTCACCAGCAAGCTTG-3′). For the other genes SuperScriptR III One-Step RT-PCR System with PlatinumR *Taq* High Fidelity kit (Invitrogen) was used with gene-specific primers to retro-transcribe and amplify trancripts in one step. We used the following primer pairs: *eomes* (5′-TCGTGGAAGTGACAGAGGAC-3′ and 5′-AGCTGGGTGATATCCGTGTT-3′), *gzma* (5′-AGGCTGTGAAAGAATCATTGGAG-3′ and 5′-ATCCTGCTACTCGGCATCTGGTTC-3′), *gzmb* (5′-ACTTTCGATCAAGGATCAGCA-3′ and 5′-ACTGTCAGCTCAACCTCTTGT-3′), *prf1* (5′-CAAGCAGAAGCACAAGTTCGT-3′ and 5′-GTGATAAAGTGCGTGCCATA -3′), *actb* (5′-GTGGGCCGCCCTAGGCACC-3′ and 5′-TCTTTGATGTCACGCACGAT-3′), *sfpi1* (5′-TCCATCGGATGACTTGGTTA -3′ and 5′-AGGAAACCTGGTGACTGAGG -3′), *zbtb46* (5′-CCACTCACTGTCTCTGAACGA-3′ and 5′-CTTCTTGTCCTTGCTGTGGA-3′).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the Intramural Research Program of the Center for Cancer Research, National Cancer Institute, National Institutes of Health. We thank Ulrich H. von Andrian for providing the B16-FLT3L melanoma cell line, Paul R. Mittelstadt for TCR-transgenic and Rémy Bosselut for Thpok-transgenic mice, respectively, Ehydel Castro for assistance with adoptive transfer, Susan H. Garfield for assistance with confocal microscopy, and Xiaolin Wu and Li Jia for help with generating and analyzing the microarray data. We also thank Rafi Ahmed for the LCMV Armstrong strain, the NIH Tetramer facility at Emory University for tetramers, and Atef Allam for LCMV preparation.

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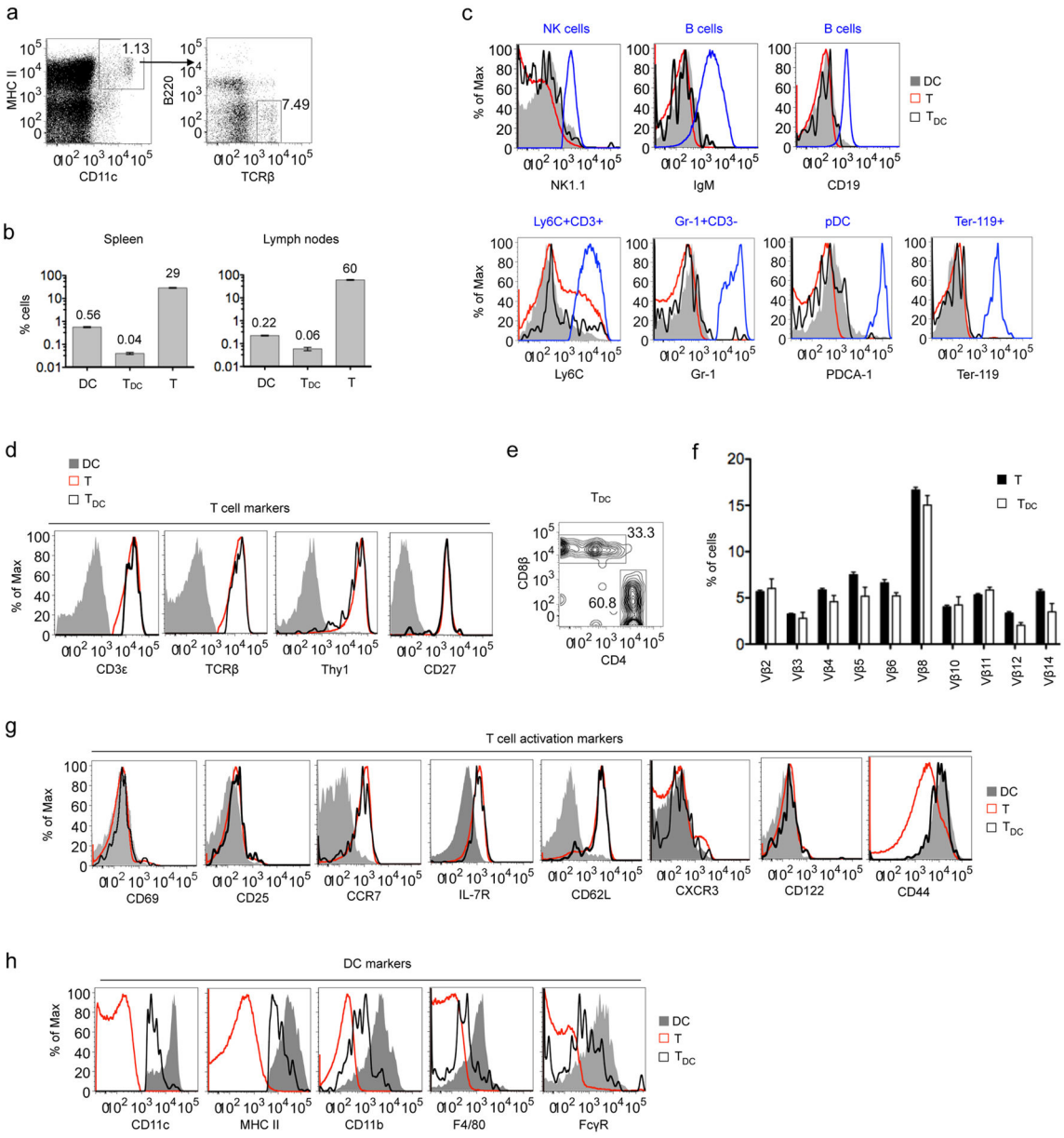

Figure 1. Identification of murine cells expressing T cell and DC markers (T_{DC})

a, Gating strategy for identification of T_{DC} from B6 splenocytes. **b,** The frequencies of the indicated subsets are shown in spleen and lymph nodes of at least ten B6 mice. Mean ± SEM is represented for each cell subset. **c–h,** Surface marker expression by the indicated subsets from B6 splenocytes. In **f**, mean ± SEM of three mice is represented for each marker.

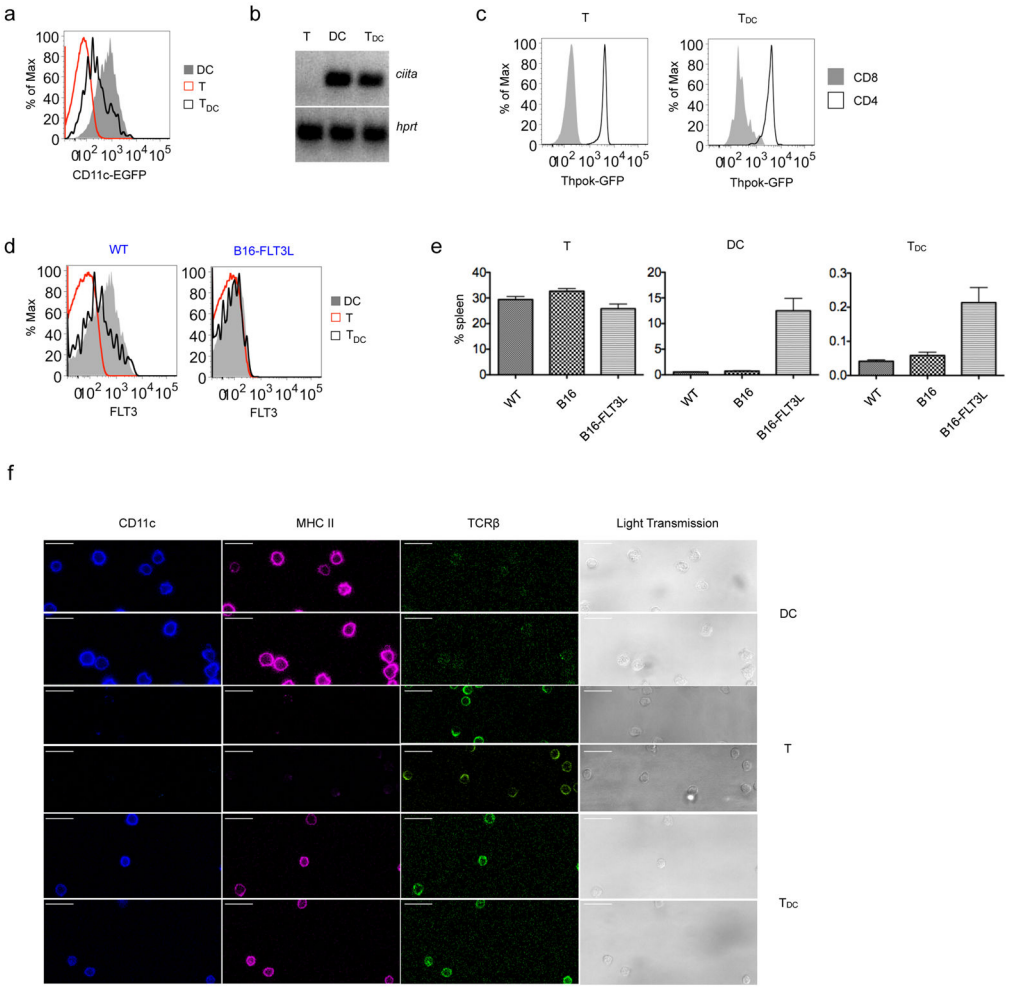

Figure 2. T_{DC} are single cells expressing T and DC molecules and expand in response to FLT3L

a,c, Fluorescence reporter expression in splenocytes of CD11c-EGFP **(a)** or Thpok-GFP **(c)** reporter mice. **b,** RT-PCR analysis of double-sorted cell subsets from B6 splenocytes. **d,e,** B6 mice were injected subcutaneously in the back with B16 or B16-FLT3L melanoma cells. Ten to thirteen days after injection the indicated splenocyte subsets were analyzed for surface FLT3 levels (**d**) or frequency (**e**). The mean ± SEM of each subset from two (B16-injected) or ten mice (WT or B16-FLT3L) is shown in **e**. Cells from uninjected mice are shown as a control (WT). **f,** Confocal microscopy analysis of cells from double-sorted splenocytes of B16-FLT3L-injected mice. Scale bars, 20 μm. One representative experiment of at least three is shown in each panel.

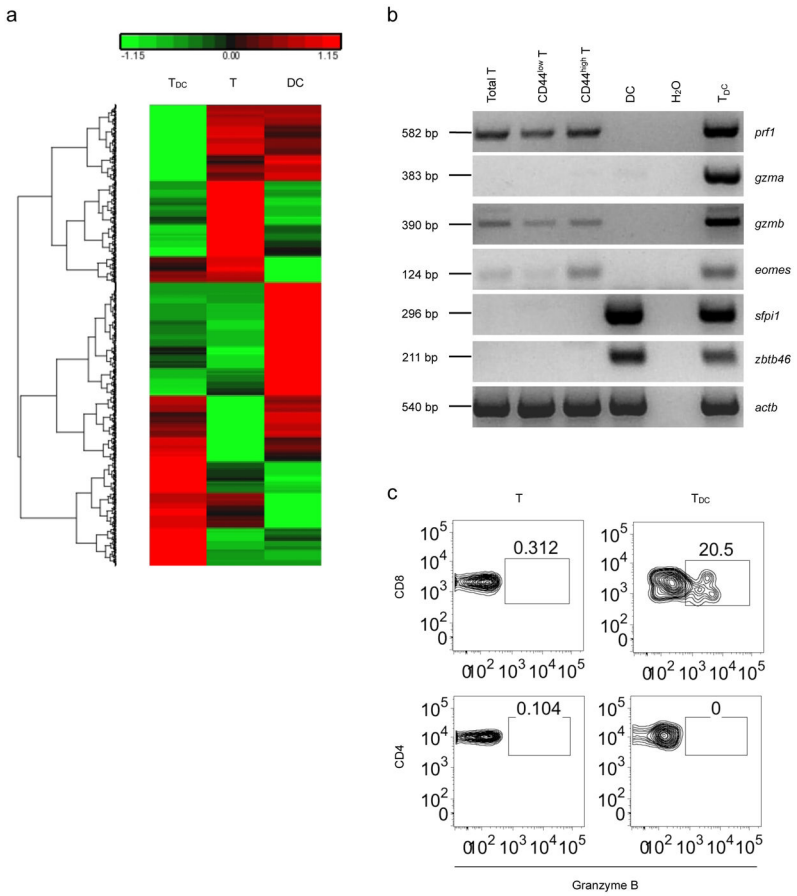

Figure 3. Molecular profiling of T_{DC} reveals a distinct genetic signature

a, Gene expression heat map from microarray analysis of highly purified cells pooled from 12 B6 mice. Individual cell subsets are represented as columns. **b,** RT-PCR of highly purified cells pooled from five B6 mice. One representative experiment of 2–4 independent analyses is shown. **c,** Flow cytometric analysis of intracellular granzyme B in splenic T or T_{DC} from B6 mice.

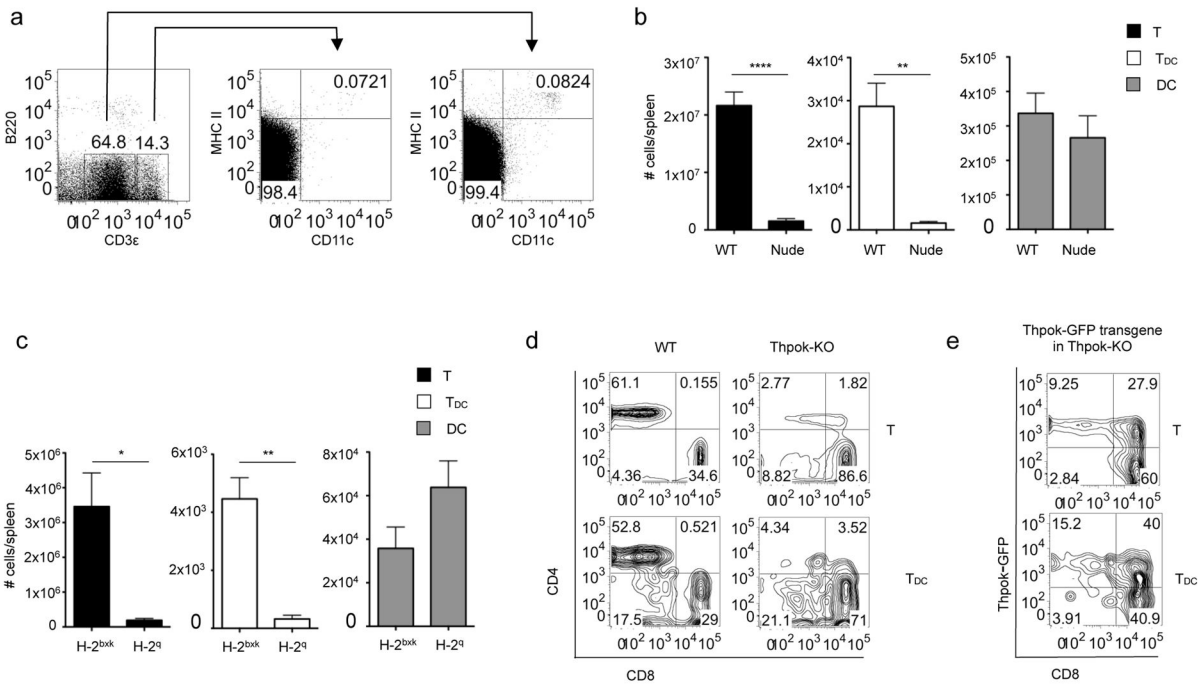

Figure 4. T_{DC} need a thymus and positive selection for development

a, Gating strategy for identification of T_{DC} in B6 thymocytes. **b–c,** Absolute numbers of the indicated cell subsets in spleens of athymic nude mice **(b)** or AND TCR Tg mice on H-2^{bxk} or H-2^{q} MHC haplotypes **(c)**. Mean ± SEM of at least 4 mice per group, statistical analysis performed with a two-tailed Student's t test. *p<0.05, **p<0.01, ****p<0.001. **d,e,** Splenocytes of B6 WT, Thpok$^{-/-}$ (**d**) or Thpok$^{-/-}$-Thpok-GFP mice (**e**) were analyzed for CD4, CD8, and GFP expression.

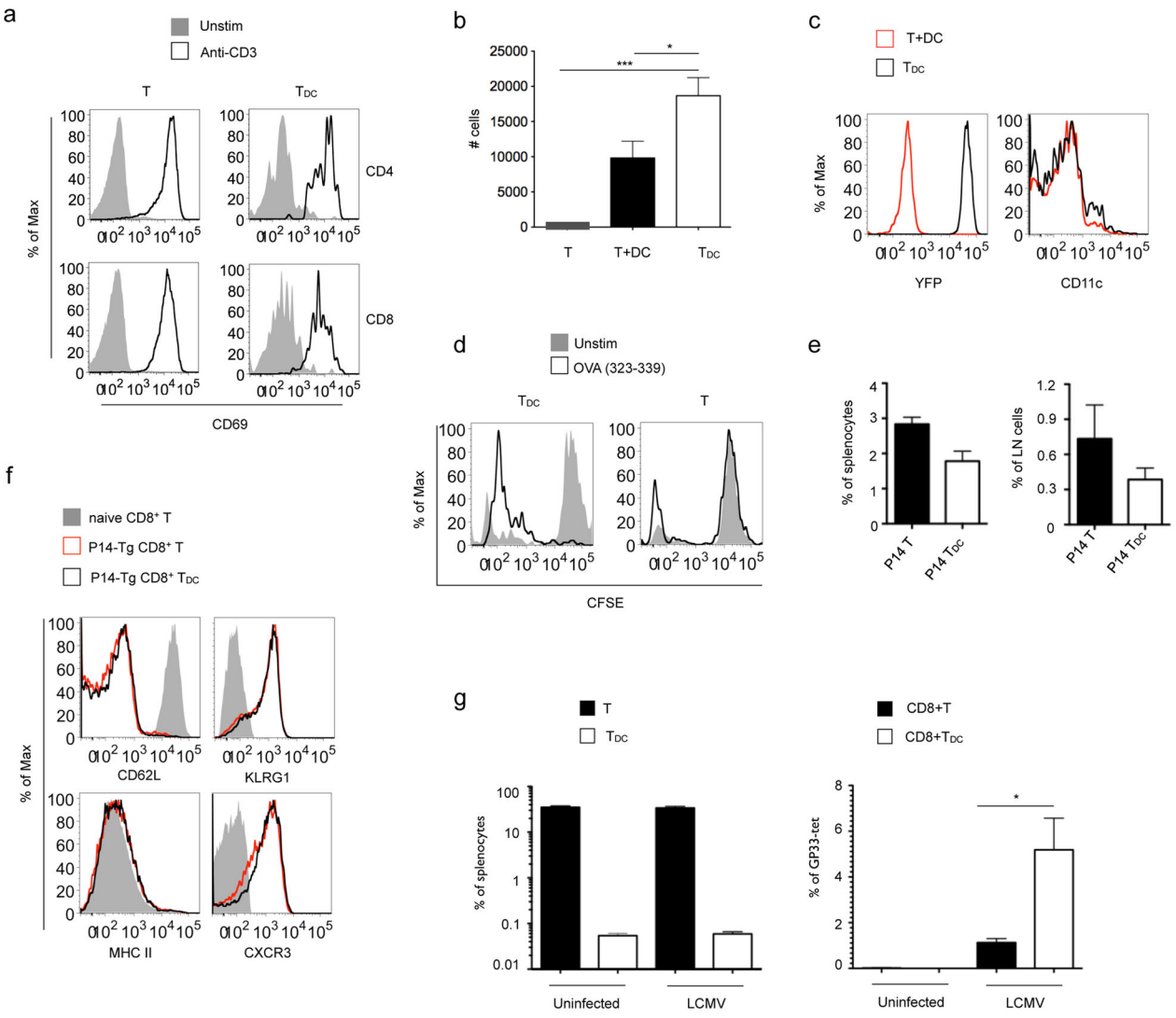

Figure 5. T_{DC} have T cell functional properties

a, Upregulation of CD69 on B6 splenocytes stimulated with soluble anti-CD3 for 18 hr. **b–c,** Cell counts and YFP expression of T_{DC} or T cells double-sorted from B6 **(b)** or Rosa26-CD11c-Cre-YFP **(c)** splenocytes, stimulated with soluble anti-CD3, supplemented with IL-2 on day 3 and analyzed on day 8. Mean ± SEM of 4 independent experiments is shown in **b. d,** CFSE dilution of double-sorted T_{DC} or T cells from OTII-Tg mice and stimulated with OVA 323-339 peptide (6.7 μM) for 7 days. **e–f,** Frequency (**e**) and surface markers (**f**) of double-sorted P14 TCR Tg T_{DC} or T cells adoptively transferred at a 1:1 ratio into B6 mice, infected the next day with LCMV Armstrong, and analysed 8 days later. Mean ± SEM of three independent experiments is shown in **e. g,** Frequency of the indicated cell subsets from B6 mice infected with LCMV Armstrong strain two months earlier. Mean ± SEM of six mice is shown, and a two-tailed Student's t test was performed. *p<0.05, ***p<0.005.

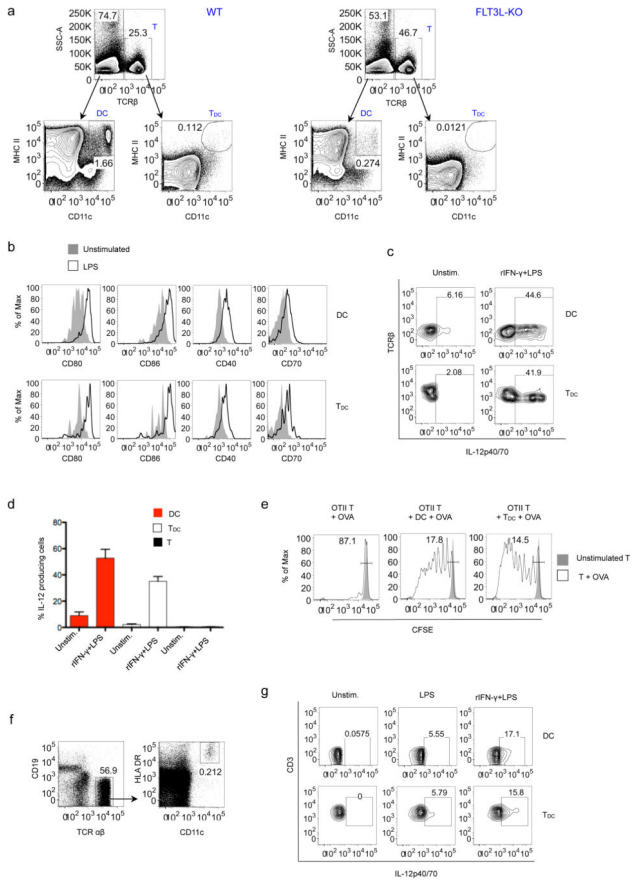

Figure 6. T_{DC} have DC properties and can be detected in human blood

a, The indicated cell subsets from WT and FLT3L-deficient mice were stained and analyzed. One representative experiment of two is shown**. b,** Flow cytometric analysis of surface costimulatory molecules in B6 gated splenic T_{DC} stimulated with LPS (100 ng/ml) for 24 hours. **c–d,** Representative experiment (**c**) or mean ± SEM of three mice (**d**) depicting IL-12 production by splenocytes of B6 mice primed with rIFN-γ (50 ng/ml) and stimulated with LPS (200 ng/ml) for 18 hours. **e**, CFSE dilution in >99% pure OTII Tg T cells co-cultured with double-sorted T_{DC} or DC stimulated with whole OVA protein (50 μg/ml). **f,** Gating strategy for identification of T_{DC} from human PBMC. **g,** Intracellular IL-12 production by human T_{DC} stimulated with LPS (200 ng/ml) for 24 hr in the presence or absence of recombinant rIFN-γ (50 ng/ml). One representative experiment of three is shown.

Table 1

Selected genes expressed by T_{DC} and DC represented as fold-difference with respect to T cells

Gene Symbol	Gene Name	T_{DC}/T	DC/T
Cd83	CD83 antigen	100	453
Itgax	CD11c	103	270
H2-Ab1	histocompatibility 2, class II antigen A, beta 1	124	199
H2-Aa	histocompatibility 2, class II antigen A, alpha	68	189
H2-Ab1	histocompatibility 2, class II antigen A, beta 1	48	150
Cd74	MHC class II-associated invariant chain	99	134
H2-Eb1	histocompatibility 2, class II antigen E beta	69	133
Flt3	fms-related tyrosine kinase 3	50	105
Irf8	interferon regulatory factor 8	17	41
Kit	kit oncogene	30	40
Ccl3	chemokine (C-C motif) ligand 3	8	39
Il12b	interleukin 12b	22	38
Lyn	tyrosine-protein kinase Lyn	16	37
Casp1	caspase-1	17	36
Ifnar1	IFN-alpha/beta receptor 1	35	33
Zbtb46	zinc finger and BTB domain containing 46	26	24
Jun	proto-oncogene c-Jun	12	24
Il13ra1	interleukin 13 receptor, alpha 1	8	14
Tlr13	toll-like receptor 13	29	14
Ciita	class II transactivator	5	12

Table 2

Selected genes expressed by T_{DC} and T cells represented as fold-difference with respect DC.

Gene Symbol	Gene Name	T_{DC}/DC	T/DC
Lck	tyrosine-protein kinase Lck	226	488
Tcrb-J	T cell receptor beta, joining region	229	366
Itk	IL2 inducible T cell kinase	210	323
Cd3d	T-cell receptor T3 delta chain	118	228
Cd247	T-cell receptor T3 zeta chain	372	175
Cd8b1	CD8 antigen, beta chain 1	58	159
Cd28	CD28 antigen	312	116
Cd27	CD27 antigen	53	108
Cd2	T-cell surface antigen CD2	89	102
Zap70	tyrosine-protein kinase ZAP-70	26	90
Thy1	thymus cell antigen 1, theta	58	89
Lat	linker for activation of T cells	45	76
Cd3g	T-cell receptor T3 gamma chain	357	72
Il2rb	interleukin 2 receptor, beta	89	69
Tcra	T cell receptor alpha chain	38	37
Foxp3	forkhead box P3	10	34
Themis	thymocyte selection associated	10	24
Notch1	Notch gene homolog 1	8	23
Ccnd2	cyclin D2	11	18
Il7r	interleukin 7 receptor	13	15