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## Inducible targeting of cDCs and their subsets *in vivo*

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## Abstract

Conventional dendritic cells (cDCs) are essential immune cells linking the innate and adaptive immune system. cDC depletion in mice is an important method to study the function of these cells *in vivo*. Here we report an inducible *in vivo* system for cDC depletion in which excision of a loxP flanked Stop signal enables expression of the human diphtheria toxin receptor (DTR) under the control of *Zbtb46* (zDC<sup>ISIDTR</sup>). cDCs can be specifically depleted by combining zDC<sup>ISIDTR</sup> mice with a *Csf1r*<sup>Cre</sup> driver line. In addition, we show that zDC<sup>Cre</sup> mice can be used to produce cDC specific conditional knockout mice (*Irf8*, *Irf4*, *Notch2*) which lack specific subsets of cDCs.

## Introduction

Expression of *Itgax* (CD11c) has been widely used as a marker for conventional dendritic cells (cDCs) and to target these cells (Caton et al., 2007; Jung et al., 2002; Lindquist et al., 2004; Stranges et al., 2007). However, pDCs, NK cells, activated monocytes, macrophages, and some lymphocytes also express CD11c limiting its use and complicating the interpretation of experiments using CD11c as a marker or for cDC depletion.

We previously described that a two-gene approach using *LysM* and *Csf1r* expression can be used to specifically deplete monocytes, monocyte-derived cells, and macrophages (Schreiber et al., 2013). The discovery of a cDC restricted transcription factor *Zbtb46* (zDC) provides an alternative means to identify and target pre-DC derived cDCs (Meredith et al., 2012; Satpathy et al., 2012). Mice that carry the green fluorescent protein under the control of the zDC gene (zDC<sup>GFP</sup>) show GFP expression specifically in cDCs (Satpathy et al., 2012) and zDC<sup>DTR</sup> mice can be used to deplete cDCs sparing other hematopoietic cells (Meredith et al., 2012). Here we describe a method for inducible depletion of cDCs; expression of the human diphtheria toxin receptor (DTR) under the control of the zDC gene is inhibited by a loxP flanked transcriptional Stop cassette (zDC<sup>ISIDTR</sup>).

Mice that express the Cre recombinase under the control of the zDC gene represent an additional tool for cDC targeting (Loschko et al., 2016). To examine

its possible uses we have combined zDC<sup>Cre</sup> mice with mice carrying loxP flanked IRF8, IRF4 and Notch2, respectively.

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## Material and methods

### Mice

zDC<sup>ISIDTR</sup> knockin mice were produced by homologous recombination in C57BL/6 albino embryonic stem cells at The Rockefeller University Gene Targeting Resource Center. The targeting strategy was the same as for the previously described zDC<sup>DTR</sup> mice (Meredith et al., 2012). zDC<sup>ISIDTR</sup> mice were maintained on an albino C57BL/6 background. Csf1r<sup>Cre</sup> BAC transgenic mice were produced by DNA microinjection into mouse oocytes. *Irf4*<sup>fl/fl</sup> (IRF4<sup>fl/fl</sup>, stock number 9380), *Irf8*<sup>fl/fl</sup> (IRF8<sup>fl/fl</sup>, stock number 14175), *Notch2*<sup>fl/fl</sup> (Notch2<sup>fl/fl</sup>, stock number 10525), *Rosa26*<sup>SIYFP</sup> (*Rosa26*<sup>SIYFP</sup>, stock number 6148) mice were purchased from The Jackson Laboratory. All mice were housed in The Rockefeller University Comparative Bioscience Center under specific pathogen free conditions. All experiments were performed in accordance with the National Institutes of Health guidelines and approved by The Rockefeller University Animal Care and Use Committee.

### DT injections

For transient cDC depletion mice were injected i.p. with 500 ng DT (Sigma) on the first day and 100 ng on all subsequent days. To analyze cell depletion mice were euthanized 16 hours after DT injection. For long-term cDC depletion DT was injected every other day.

### Flow cytometry

For FACS analysis, cells were stained using antibodies against B220 (RA3-6B2), BST2 (eBio927), F4/80 (BM8), Gr-1 (RB6-8C5), Ly6C (AL-21), Ly6G (1A8), MHCII (M5/114.15.2), NK1.1 (PK136), Siglec-H (440c), CD3 (145-2C11), CD4 (RM4-5), CD8 (53-6.7), CD11b (M1/70), CD11c (N418), CD19 (1D3), CD115 (AFS98). PI (Sigma), DAPI (Invitrogen) or Aqua live/dead (Invitrogen) was added to exclude dead cells from analysis. Cells were analyzed on a LSR Fortessa flow cytometer (BD Biosciences). Analysis was performed using FlowJo software (Tree Star).

### **BM and spleen cell isolation**

For isolation of mononuclear phagocytes spleens were mechanically disrupted and incubated with HBSS (Gibco) + 5% FBS + 0.5 mg/mL CollagenaseD (Roche) at 37 °C for 30 min. Cells were filtered through 100 µM cell strainers and washed. Erythrocytes were lysed with ACK Lysing buffer (Gibco). BM cells were isolated from femurs and tibiae and filtered through 100 µM cell strainers. Erythrocytes were lysed with ACK Lysing buffer (Gibco).

### **ELISA**

Serum levels of Flt3L were determined by ELISA (R&D Systems) according to the manufacturer's protocol

### **Statistical Analysis**

Results are shown as a means + SEM. Comparisons between groups were done using Student's two-tailed *t* test analysis. Statistical significance was determined as *P* values <0.05.

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## Results

### The MNP lineage

Dendritic cells (DCs), monocytes, and macrophages are closely related and together comprise the mononuclear phagocyte (MNP) system. In mice the macrophage-DC progenitor (MDP) in the bone marrow (BM) gives rise to monocytes and a common DC progenitor (CDP). Monocytes circulate in the blood and peripheral tissues where they can be activated and develop into “inflammatory” DCs and some but not all types of macrophages (Hashimoto et al., 2013). CDPs give rise to plasmacytoid DCs (pDCs) and pre-DCs, progenitor cells that are committed to become cDCs (Liu et al., 2009). Both pDCs and pre-DCs leave the BM and seed lymphoid and non-lymphoid tissues (Geissmann et al., 2010). Pre-DCs differentiate into cDCs that can be divided into CD8 $\alpha^+$  and CD11b $^+$  cDCs, respectively (Fig. 1 and (Hildner et al., 2008; Lewis et al., 2011; Tamura et al., 2005)). In mice, pre-DCs appear to be heterogeneous and can be divided into cells committed to become CD8 $\alpha^+$  or CD11b $^+$  cDCs, respectively (Grajales-Reyes et al., 2015; Schlitzer et al., 2015).

### zDC<sup>ISIDTR</sup> and Csf1r<sup>Cre</sup> mice

zDC is expressed in cDCs and their immediate committed precursors but not in other cells of the MNP system (Fig. 1 and (Meredith et al., 2012; Satpathy et al., 2012)). zDC has been used to deplete (zDC<sup>DTR</sup>), label (zDC<sup>GFP</sup>) and target (zDC<sup>Cre</sup>) cDCs (Loschko et al., 2016; Meredith et al., 2012; Satpathy et al., 2012). However, zDC is also expressed in endothelial cells. Therefore, prolonged cDC

depletion in zDC<sup>DTR</sup> mice by diphtheria toxin (DT) injection requires the use of bone marrow chimeras (BMC). To produce an inducible zDC<sup>DTR</sup> mouse, we inserted a loxP flanked transcriptional Stop element in front of the DTR, and inserted the cassette into the zDC gene (zDC<sup>ISIDTR</sup>) (Fig. 2). Cre mediated excision of the Stop element results in DTR expression under the control of the zDC promoter.

To restrict DTR expression to cDCs in zDC<sup>ISIDTR</sup> mice, we chose BAC transgenic mice expressing Cre under the control of the Csf1 receptor gene (Csf1r<sup>Cre</sup>, Fig. 3A). Csf1r is expressed in monocytes, macrophages, and DC precursors (MDP and CDP) (Fig. 1). To determine the specificity of Cre expression in Csf1r<sup>Cre</sup> mice we crossed Csf1r<sup>Cre</sup> to Rosa26<sup>ISiYFP</sup> mice. Because pre-DCs descend from Csf1r+ CDPs we reasoned that pre-DCs in the BM as well as in the spleen of Csf1r<sup>Cre</sup>Rosa26<sup>ISiYFP</sup> mice should be labeled with YFP. As expected, all pre-DCs and cDCs were YFP<sup>+</sup> (Fig. 3B). In addition, we found YFP expression in pDCs, monocytes and macrophages in Csf1r<sup>Cre</sup>Rosa26<sup>ISiYFP</sup> mice since these cells also descend from Csf1r<sup>+</sup> precursor cells or express Csf1r, respectively (Fig. 3B). The percentage of labeled pDCs was lower than in cDCs, macrophages and monocytes possibly because pDCs can develop from myeloid as well as lymphoid precursors (Sathé et al., 2013). Thus, although Cre expression in Csf1r<sup>Cre</sup> mice is not specific for cDCs, cDCs are efficiently targeted in these mice (near 100% recombination efficiency) and therefore Csf1r<sup>Cre</sup> mice are suitable for cDC targeting.

**Csf1r<sup>Cre</sup>zDC<sup>ISIDTR</sup> mice**

Hemizygous Csf1r<sup>Cre</sup> were bred to homozygous zDC<sup>ISIDTR</sup> mice to obtain Cre<sup>+</sup> (Csf1r<sup>Cre+</sup>zDC<sup>ISIDTR</sup>) and Cre<sup>-</sup> control (Csf1r<sup>Cre-</sup>zDC<sup>ISIDTR</sup>) littermates (Fig. 4A). 16 hours after DT administration we found efficient depletion of cDCs but not monocytes, macrophages, CD8<sup>+</sup> T or B cells (Fig. 4B). We observed slightly lower levels of pDCs and CD4<sup>+</sup> T cells in Csf1r<sup>Cre+</sup>zDC<sup>ISIDTR</sup> mice (Fig. 4B). However, we believe that these cells were not directly depleted by DT as they do not express zDC and hence do not express the DTR.

Expression of zDC in endothelial cells requires the use of BMC to deplete cDCs in zDC<sup>DTR</sup> mice (Meredith et al., 2012); otherwise zDC<sup>DTR</sup> mice die 24-48 hours after DT injection (Meredith et al., 2012). In Csf1r<sup>Cre+</sup>zDC<sup>ISIDTR</sup> mice expression of DTR under the control of zDC is restricted to cells that descend from Csf1r expressing precursors. This approach omits endothelial cells. Among hematopoietic cells cDCs are the only cells expressing zDC and therefore DTR expression in Csf1r<sup>Cre+</sup>zDC<sup>ISIDTR</sup> mice is restricted to these cells. Therefore cDCs can be efficiently depleted for at least 4 weeks directly in these mice without the requirement to produce BMC (Fig. 4C).

As previously demonstrated cDC depletion results in elevated levels of Flt3L (Fig. 4D and (Birnberg et al., 2008; Meredith et al., 2012)); this has been associated with a myeloproliferative disorder (Birnberg et al., 2008). We also observed

elevated levels of neutrophils and monocytes in the spleen of mice depleted of cDCs for 4 weeks (Fig. 4E). This “side-effect” has to be kept in mind when depleting cells for a prolonged period of time. We conclude that  $Csf1r^{Cre+}zDC^{ISIDTR}$  mice can be used to efficiently deplete cDCs *in vivo*.

### **cDC specific conditional knockout mice lacking cDC subsets**

cDCs can be divided into two subsets; IRF8/Batf3 dependent  $CD8\alpha^+$  cDCs and IRF4/Notch2 dependent  $CD11b^+$  DCs (Hildner et al., 2008; Lewis et al., 2011; Tamura et al., 2005). To test whether the two cDC subsets can be depleted using  $zDC^{Cre}$  mice we crossed them with  $IRF8^{fl/fl}$ ,  $IRF4^{fl/fl}$  or  $Notch2^{fl/fl}$  mice and compared  $Cre^-$  and  $Cre^+$  littermates.  $zDC^{Cre+}IRF8^{fl/fl}$  mice had normal frequencies of pDCs and cDCs in the spleen (Fig. 5A, B). However, they had significantly fewer  $CD8\alpha^+/DEC205^+$  cDCs compared to their  $Cre^-$  littermates (Fig. 5A, C). We conclude that  $zDC^{Cre}$  mice can be used to conditionally delete *Irf8* expression in cDCs to produce mice deficient in the  $CD8\alpha^+/DEC205^+$  cDC subset.

In contrast,  $zDC^{Cre+}Notch2^{fl/fl}$  as well as  $zDC^{Cre+}IRF4^{fl/fl}$  mice did not show reduced frequencies of  $CD8\alpha^+$  cDCs. However, we detected lower percentages of  $CD11b^+$  cDCs in these mice. Some  $CD11b^+$  cDCs also express CD4, ESAM and DCIR2, respectively. We found fewer cells expressing these markers in  $zDC^{Cre+}Notch2^{fl/fl}$  and  $zDC^{Cre+}IRF4^{fl/fl}$  mice indicating that Notch2 and IRF4 are required for full differentiation of  $CD11b^+$  cDCs (Fig. 6A, C, D, F). In  $IRF4^{fl/fl}$  mice expression of GFP marks cells with recombined loxP sites (Klein et al., 2006).

Analyzing GFP expression showed that in the majority of cDCs loxP sites recombined. Therefore, the reason for incomplete absence of CD11b<sup>+</sup> cDCs cannot be inefficient recombination and IRF4 deletion. More likely Cre mediated deletion of IRF4 happens at a time during cDC differentiation where IRF4 is already at least partially dispensable.

We conclude that zDC<sup>Cre</sup> mice can be used to deplete CD8 $\alpha$ <sup>+</sup> cDCs (zDC<sup>Cre+IRF8<sup>fl/fl</sup></sup>) and produce mice that show reduced frequencies albeit not complete deletion of CD11b<sup>+</sup> cDCs (zDC<sup>Cre+Notch2<sup>fl/fl</sup></sup> and zDC<sup>Cre+IRF4<sup>fl/fl</sup></sup>). Whether the remaining CD11b<sup>+</sup> cDCs in zDC<sup>Cre+Notch2<sup>fl/fl</sup></sup> and zDC<sup>Cre+IRF4<sup>fl/fl</sup></sup> mice show functional impairment has to be investigated.

## Discussion

Gene targeting is an essential tool to study the function of cDCs *in vivo*. A frequent limitation of these *in vivo* models is partial specificity. Until recently CD11c expression was the most specific marker to target cDCs. CD11c has been used to label (CD11c<sup>YFP</sup>), deplete (CD11c<sup>DTR</sup>), and conditionally target (CD11c<sup>Cre</sup>) cDCs (Caton et al., 2007; Jung et al., 2002; Lindquist et al., 2004; Stranges et al., 2007). However, CD11c expression is not restricted to cDCs but also found in pDCs, activated monocytes, macrophages and some NK cells. (Hohl et al., 2009; Meredith et al., 2012; Schreiber et al., 2013; Serbina et al., 2003).

Among hematopoietic cells expression of zDC is restricted to cDCs (Meredith et al., 2012; Satpathy et al., 2012). However, endothelial cells express zDC as well (Satpathy et al., 2012), and therefore zDC<sup>DTR</sup> mice die after DT administration. The inducible zDC<sup>ISIDTR</sup> mouse described here allows prolonged depletion of cDCs in non-BMC mice, which is a significant improvement. The same inducible zDC<sup>ISIDTR</sup> mouse can also be used to deplete cDC subsets when combined with Cre driver lines that are specific for CD8 $\alpha$ <sup>+</sup> and CD11b<sup>+</sup> cDCs, respectively. Potential candidate genes include *Xcr1*, *Ly75* (DEC205), *Itgam* (CD11b), and *Clec4a4* (Dcir2). The available CD4<sup>Cre</sup> and CD8<sup>Cre</sup> mice have been tested but proved to be not useful for targeting cDCs (data not shown).

An alternative approach to deplete cDC subsets is to produce cDC specific conditional knockouts. Mice lacking IRF8 in cDCs ( $zDC^{Cre+IRF8^{fl/fl}}$ ) for example lack  $CD8\alpha^+$  cDCs. Although *Batf3* deficient mice also lack  $CD8\alpha^+$  cDCs (Hildner et al., 2008) compensatory mechanisms can lead to *Batf3* independent  $CD8\alpha^+$  cDC development (Tussiwand et al., 2012).  $zDC^{Cre+IRF8^{fl/fl}}$  mice provide a valuable tool to study the contribution of  $CD8\alpha^+$  cDCs during immune activation (Esterhazy et al., 2016).

Although IRF4 deficient mice lack  $CD4^+$  cDCs (Tamura et al., 2005)  $zDC^{Cre+Notch2^{fl/fl}}$  and  $zDC^{Cre+IRF4^{fl/fl}}$  mice are only partially deficient in  $CD11b^+/CD4^+$  cDCs. This discrepancy could be due to timing of deletion. In  $zDC^{Cre+Rosa^{ISiyFP}}$  mice pre-DCs are YFP<sup>-</sup> (data not shown) whereas pre-DCs in  $zDC^{GFP}$  mice are GFP<sup>+</sup> (Satpathy et al., 2012). We speculate that by the time the loxP sites have recombined and YFP starts getting expressed the cells already became MHCII<sup>+</sup> cDCs. In case of  $zDC^{Cre+Notch2^{fl/fl}}$  and  $zDC^{Cre+IRF4^{fl/fl}}$  mice this would mean that differentiation of cDCs might be complete before deletion of *Notch2* and *IRF4*, respectively and that *zDC* expressing cells are not dependent on *Notch2* or *IRF4* for their final differentiation. Another explanation for the presence of  $CD11b^+$  cDCs in  $zDC^{Cre+Notch2^{fl/fl}}$  and  $zDC^{Cre+IRF4^{fl/fl}}$  mice could be incomplete deletion or that mRNA and/or protein have a long half-life. *IRF4* deficient cDCs were described to be functionally impaired (Vander Lugt et al., 2014). Whether  $CD11b^+$  cDCs are functionally defective in  $zDC^{Cre+IRF4^{fl/fl}}$  mice remains to be investigated.

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**Conflict of interest**

The authors report no competing financial interests.

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**Fig. 1.** The MNP network. The macrophage-DC progenitor (MDP) resides in the bone marrow and gives rise to monocytes (mono) and common DC progenitors (CDP). Monocytes circulate in the blood and can differentiate into “inflammatory” DCs and some but not all macrophages after activation. CDPs are restricted to become pDCs or pre-DCs. Both cells seed peripheral tissues. pre-DCs become CD8 $\alpha$ <sup>+</sup> and CD11b<sup>+</sup> cDCs, respectively. Cells expressing Csf1r and zDC, respectively, are labeled accordingly.

**Fig. 2.** Generation of the zDC<sup>ISIDTR</sup> mouse. Schematic diagram of the *Zbtb46* (*zDC*) locus. 5' and 3' UTRs are shown in black, coding regions in white. A transcriptional Stop element flanked by loxP sites and followed by the sequence encoding for IRES-DTR-mCherry was introduced in the 3'UTR of *zDC* right after the endogenous Stop codon. After Cre mediated recombination of loxP sites the DTR-mCherry fusion protein is expressed under the control of the zDC promoter.

**Fig. 3.** Fate mapping using a BAC transgenic *Csf1r*<sup>Cre</sup> mouse. (A) Schematic diagram of the *Csf1r* locus. 3' UTR is shown in black, coding regions in white. To express Cre recombinase in cells that actively transcribe *Csf1r*, we inserted IRES-Cre into the 3' UTR of *Csf1r* right after the endogenous Stop codon. (B) Dot plots show gating strategy in the spleen to define pDCs, cDCs, pre-DCs, RPMs and monocytes. pDCs (BST2<sup>+</sup>CD11c<sup>+</sup>), cDCs (BST2<sup>-</sup>Lin<sup>-</sup>CD11c<sup>high</sup>MHCII<sup>+</sup>), pre-DCs (BST2<sup>-</sup>Lin<sup>-</sup>CD11c<sup>+</sup>MHCII<sup>-</sup>Flt3<sup>+</sup>Sirp $\alpha$ <sup>-</sup>), RPM (Lin<sup>-</sup>CD11b<sup>low</sup> F4/80<sup>+</sup>), monocytes (Lin<sup>-</sup>CD11b<sup>+</sup>F4/80<sup>-</sup>Ly6G<sup>-</sup>Ly6C<sup>+</sup>CD115<sup>+</sup>).

Histograms show percentage of cells expressing YFP in  $Csf1r^{Cre}Rosa^{ISiYFP}$  mice (open histograms) or  $Rosa^{ISiYFP}$  control mice (filled grey histograms). Graph depicts percentage of indicated cells expressing YFP.

**Fig. 4.**  $Csf1r^{Cre}zDC^{SIDTR}$  mice. (A) Hemizygous  $Csf1r^{Cre}$  mice were bred with homozygous  $zDC^{SIDTR}$  mice. Resulting offspring consists of  $Cre^{-}$  control ( $Csf1r^{Cre-}zDC^{SIDTR}$ ) and  $Cre^{+}$  ( $Csf1r^{Cre+}zDC^{SIDTR}$ ) mice. (B) 500 ng DT was injected i.p.; 16 hours later spleens were isolated and cDC depletion was determined by flow cytometry. Graphs depict percentage of pDCs, cDCs, monocytes, macrophages, T and B cells among living splenocytes. Each data point represents one individual mouse (n=5-8). (C, D) Mice received 500 ng DT the first day and 100 ng every other day thereafter for 4 weeks. (C) Graph depicts percentage of pDCs and cDCs among living splenocytes. Each data point represents one individual mouse (n=10-11) (D) Graph depicts serum levels of Flt3L in pg/mL. Each data point represents one individual mouse (n=10-11). (E) Graphs depict percentage of neutrophils and monocytes among living splenocytes. Each data point represents one individual mouse (n=10-11). \* P value < 0.05

**Fig. 5.**  $zDC^{Cre}IRF8^{fl/fl}$  mice. (A) Flow cytometric analysis of splenocytes from  $Cre^{-}$  ( $zDC^{Cre-}IRF8^{fl/fl}$ ) and  $Cre^{+}$  ( $zDC^{Cre+}IRF8^{fl/fl}$ ) mice. (B) Graph depicts frequency of pDCs and cDCs among living splenocytes. (C) Graph depicts ratio of  $CD8\alpha^{+}$ ,  $DEC205^{+}$  and  $CD11b^{+}$  cDCs. Each data point represents one individual mouse (n=9). \* P value < 0.05

**Fig. 6.** zDC<sup>Cre</sup>Notch2<sup>fl/fl</sup> and zDC<sup>Cre</sup>IRF4<sup>fl/fl</sup> mice. (A) Flow cytometric analysis of splenocytes from Cre<sup>-</sup> (zDC<sup>Cre-</sup>Notch2<sup>fl/fl</sup>) and Cre<sup>+</sup> (zDC<sup>Cre+</sup>Notch2<sup>fl/fl</sup>) mice. (B) Graph depicts frequency of pDCs and cDCs among living splenocytes. Each data point represents one individual mouse (n=8). (C) Graph depicts ratio of CD8 $\alpha$ <sup>+</sup>, CD11b<sup>+</sup>, CD4<sup>+</sup>, ESAM<sup>+</sup> and DCIR2<sup>+</sup> cDCs. Each data point represents one individual mouse (n=7-8). (D) Flow cytometric analysis of splenocytes from Cre<sup>-</sup> (zDC<sup>Cre-</sup>IRF4<sup>fl/fl</sup>) and Cre<sup>+</sup> (zDC<sup>Cre+</sup>IRF4<sup>fl/fl</sup>) mice. (E) Graph depicts frequency of pDCs and cDCs among living splenocytes. Each data point represents one individual mouse (n=10). (F) Graph depicts ratio of CD8 $\alpha$ <sup>+</sup>, CD11b<sup>+</sup>, CD4<sup>+</sup>, ESAM<sup>+</sup> and DCIR2<sup>+</sup> cDCs. Each data point represents one individual mouse (n=6-10). \* P value < 0.05

Figure 1

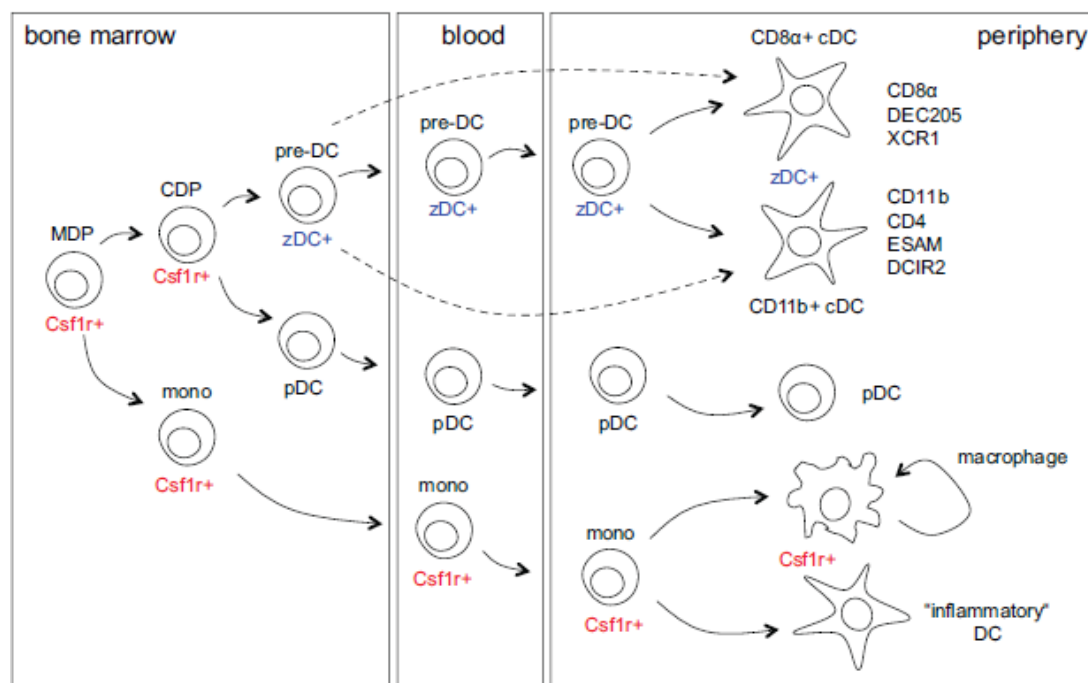


Figure 2

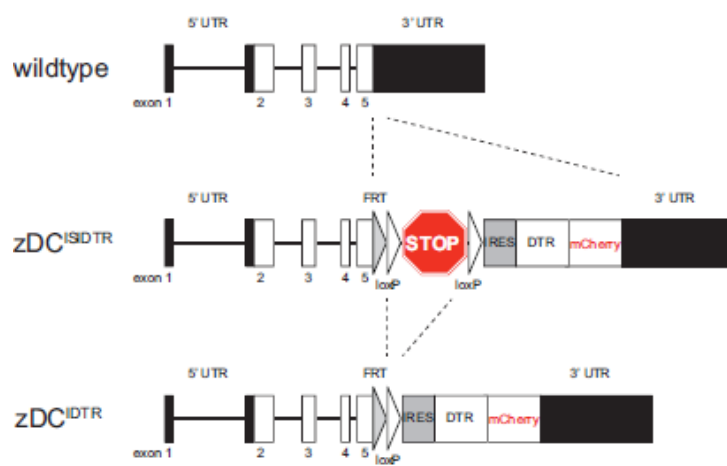


Figure 2

Figure 3

Figure 3

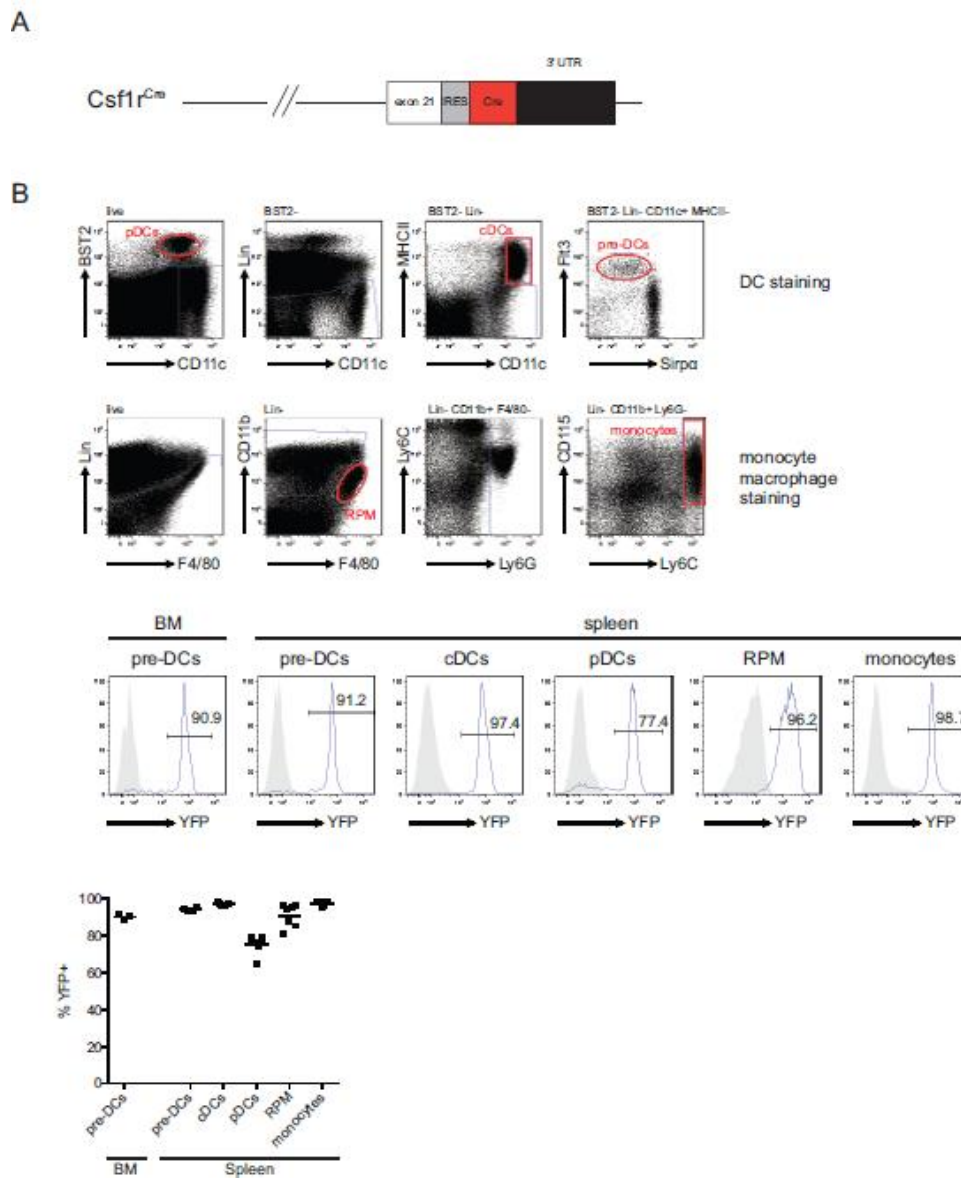


Figure 4

Figure 4

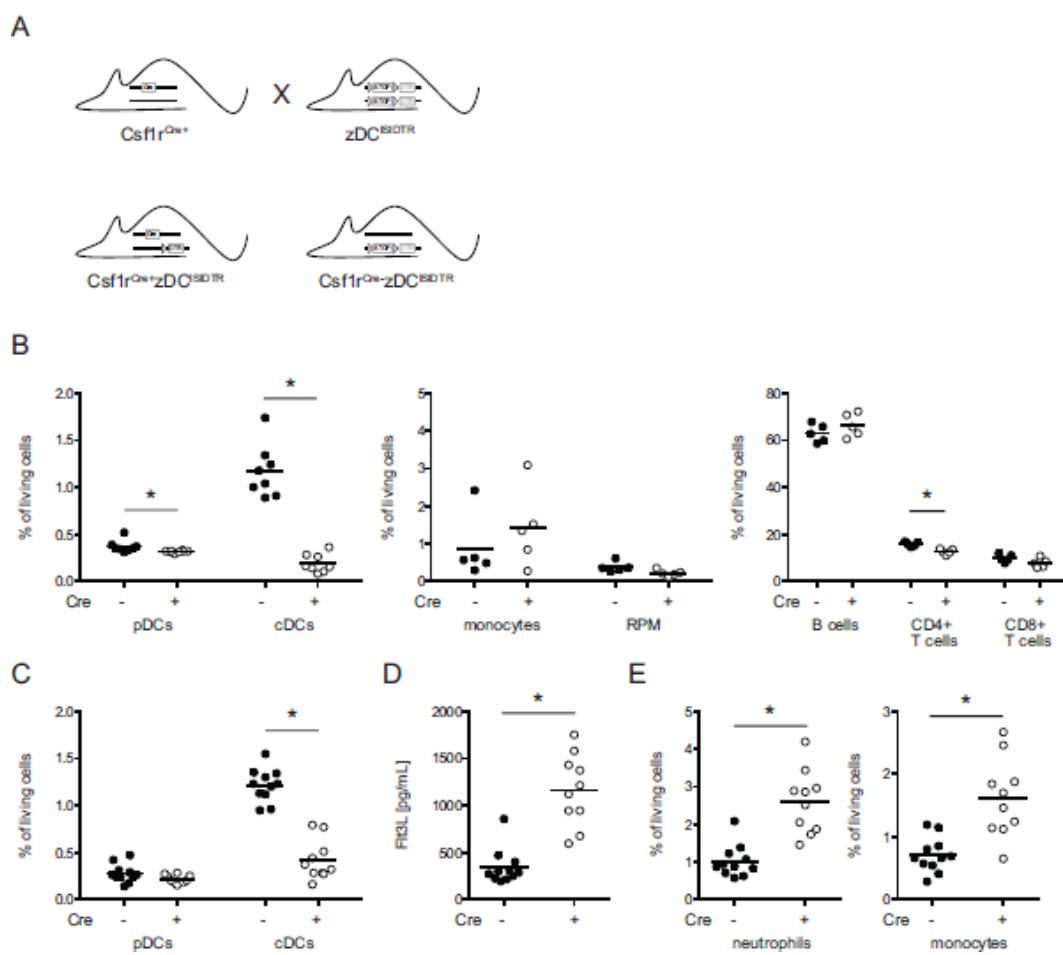


Figure 5

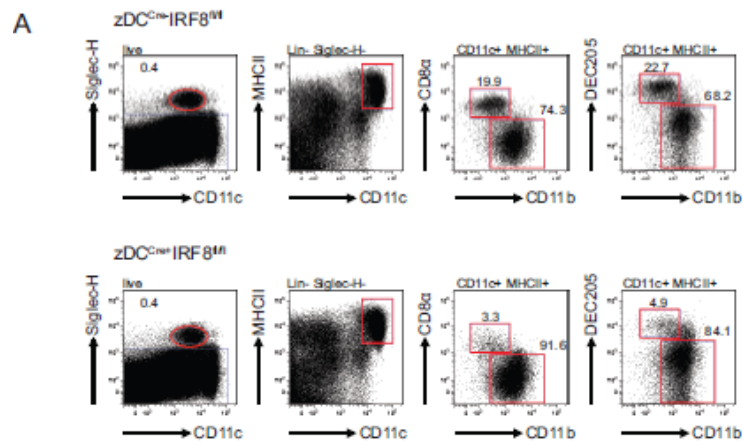


Figure 5

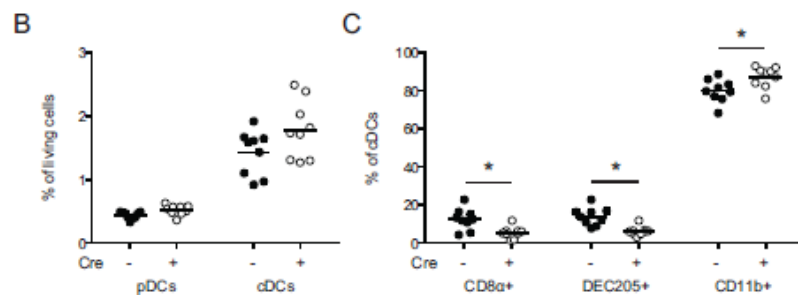


Figure 6

