# IL7Rα, but not Flk2/Flt3, is required for hematopoietic stem cell reconstitution of tissue-resident lymphoid cells

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## SUMMARY STATEMENT

Tissue-resident lymphoid cells (TLCs) develop via IL7Rα-positive progenitors and are repopulated by transplanted adult hematopoietic stem cells (HSCs); however, HSCs cannot fully rescue TLC lymphopoiesis in IL7Rα$^{-/-}$ recipient mice.

# ABSTRACT

Tissue-resident lymphoid cells (TLCs) span the spectrum of innate-to-adaptive immune function. Unlike traditional, circulating lymphocytes that are continuously generated from hematopoietic stem cells (HSCs), many TLCs are of fetal origin and poorly generated from adult HSCs. Here, we sought to further understand murine TLC development and the roles of Flk2 and IL7Rα, two cytokine receptors with known function in traditional lymphopoiesis. Using Flk2- and Il7r-Cre lineage tracing, we found that peritoneal B1a cells, splenic marginal zone B (MZB) cells, lung ILC2s and regulatory T cells (Tregs) were highly labeled. Despite high labeling, loss of Flk2 minimally affected the generation of these cells. In contrast, loss of IL7Rα, or combined deletion of Flk2 and IL7Rα, dramatically reduced the number of B1a cells, MZBs, ILC2s, and Tregs both *in situ* and upon transplantation, indicating an intrinsic and essential role for IL7Rα. Surprisingly, reciprocal transplants of WT HSCs showed that an IL7Rα$^{-/-}$ environment selectively impaired reconstitution of TLCs when compared to TLC numbers *in situ*. Taken together, our data defined Flk2- and IL7Rα-positive TLC differentiation paths, and revealed functional roles of Flk2 and IL7Rα in TLC establishment.


# INTRODUCTION

Traditional, circulating immune cells are typically defined as either myeloid or lymphoid and generated from hematopoietic stem cells (HSCs). Myeloid cells are involved in rapid, broad response innate immunity whereas traditional lymphoid cells are involved in slow, specific adaptive immunity (Bonilla and Oettgen, 2010; Netea, Quintin and van der Meer, 2011; Beaudin and Forsberg, 2016; Cool and Forsberg, 2019). The adaptive immune response relies on the complex B and T cell receptor repertoire generation. Although this dichotomy of immune cells is clear for circulating cells, it is unclear if tissue-resident immune cells squarely belong to the myeloid or lymphoid lineage with many recent studies alluding to complex and dynamic origins (Beaudin and Forsberg, 2016; Beaudin *et al.*, 2016; Leung *et al.*, 2019). Unlike circulating immune cells that traffic to non-lymphoid organs upon activation, tissue-resident immune cells reside in

non-lymphoid organs, do not recirculate, and have specialized functions that span the innate-adaptive spectrum (Davies *et al.*, 2013; Chou and Li, 2018). For example, B1a cells in the peritoneal cavity are considered B cells (Kantor and Herzenberg, 1993). However, they do not undergo the same B cell receptor selection process as circulating B cells and are thus deemed an “innate-like” lymphoid cell. While recent studies have rapidly propelled the understanding of tissue macrophage specification and function (Blériot *et al.*, 2020; Martin and Gurevich, 2021), it is unclear if TLC differentiation is orchestrated similarly to circulating lymphoid cells; here we sought to determine if their differentiation is regulated by classic lymphoid genes.

We were particularly interested in the cytokine receptors Flk2 and IL7Rα because they are required for traditional adult lymphopoiesis. This finding was demonstrated by impaired lymphopoiesis in both Flk2$^{-/-}$ and Il7rα$^{-/-}$ mice and supported by Flk2-Cre and Il7rα-Cre driven lineage tracing of cells with increasingly restricted lymphoid potential (Peschon *et al.*, 1994; Kikuchi *et al.*, 2008; Beaudin, Boyer and Forsberg, 2014; Leung *et al.*, 2019) **(Fig. 1A, 1B, 1C)**. As previously described (Boyer *et al.*, 2011; Boyer, Beaudin and Forsberg, 2012; Beaudin *et al.*, 2016; Leung *et al.*, 2019; Cool *et al.*, 2020), these models were generated by crossing mice expressing Flk2-Cre (Benz *et al.*, 2008) or Il7r-Cre (Schlenner *et al.*, 2010) to mTmG mice expressing a dual-color fluorescent reporter (Muzumdar *et al.*, 2007) creating the “FlkSwitch” and “Il7rSwitch” models (**Fig.1A**). In both models, cells express Tomato (Tom) until Cre-mediated recombination results in the irreversible switch to GFP expression by that cell and all of its downstream progeny (**Fig.1B, 1C**). We previously demonstrated that Il7r-Cre labeling is not constrained to lymphoid cells. Surprisingly, in the “Il7rSwitch” lineage tracing model, fetally-derived adult tissue-resident macrophages (TrMacs), were highly labeled and IL7Rα was required for early TrMac development (Leung *et al.*, 2019), whereas Flk2-Cre did not efficiently label TrMacs (Hoeffel *et al.*, 2011; Hashimoto *et al.*, 2013; Epelman *et al.*, 2014; Leung *et al.*, 2019). We also previously examined lung eosinophils, another myeloid cell type; interestingly, despite the high Flk2-Cre and minimal Il7r-Cre labeling of eosinophils, they depend on cell extrinsic IL7Rα, but not Flk2 (Cool *et al.*, 2020). The development and generation of these tissue-resident

myeloid cells evidently does not follow that of other traditional, circulating myeloid cells. Similarly, many innate-like lymphoid cells are thought to arise from fetal progenitors (Beaudin and Forsberg, 2016) and it remains unclear if their development follow traditional lymphoid paths and whether they are functionally regulated by known lymphoid drivers. For example, there is evidence that many TLCs arise via common lymphoid progenitor (CLP)-independent pathways (Ghaedi *et al.*, 2016), and there is differential requirement for Flk2 and IL7Rα amongst different hematopoietic cell types (Sitnicka *et al.*, 2007; Beaudin, Boyer and Forsberg, 2014). To determine whether Flk2 and IL7Rα are involved in TLC development, we employed lineage tracing, germline knockouts, and HSC transplantation assays.

# RESULTS

## Flk2-Cre and Il7r-Cre highly label tissue-resident lymphoid cell populations

To test whether TLCs arise via differentiation pathways similar to circulating lymphocytes, we examined labeling of TLCs in the FlkSwitch and Il7rSwitch lineage tracing models (**Fig. 1A-C**). We compared reporter expression of traditional peripheral blood B cells to reporter expression of B1a cells isolated from the peritoneal cavity, marginal zone B cells (MZB) from the spleen, type 2 innate lymphoid cells (ILC2) from the lung, and regulatory T cells (Treg) from the lung (**Fig. 1D-H**). As expected, and previously reported, Cre-driven labeling of circulating peripheral blood B cells was greater than 95% in both FlkSwitch and Il7rSwitch mice (**Fig. 1D**) (Schlenner *et al.*, 2010; Leung *et al.*, 2019). Similarly, Cre-driven labeling was uniformly high for all TLCs examined, a trend we also observed in B1b and B2 cells (**Fig. S1A**). Together, our data showed that B1a cells, ILC2, MZB and Tregs arise from Flk2- and IL7Rα-positive cells, as do traditional circulating lymphoid cells.

## Tissue-resident lymphoid cells are severely reduced in the absence of IL7Rα, but not Flk2

We and others have previously shown that loss of Flk2 results in a reduction of hematopoietic progenitor cells, with a less severe reduction in mature B cells in the peripheral blood (Mackarehtschian *et al.*, 1995; Sitnicka *et al.*, 2007; Jensen *et al.*, 2008; Beaudin, Boyer and Forsberg, 2014). The loss of IL7Rα has also been shown to result in a severe reduction of peripheral blood B cells (Peschon *et al.*, 1994). Here, we observed similar trends in the lung, where traditional CD19+ B cells, although not significantly reduced in Flk2$^{-/-}$ mice, were significantly reduced in the IL7Rα$^{-/-}$ mice (**Fig. 2A,2B**). Similar trends were observed in peripheral blood CD3+ T cells (**Fig S2A**). To determine if TLCs were similarly affected, we quantified their cellularity in the absence of Flk2 and IL7Rα. Compared to WT numbers of each cell type, only MZBs were significantly reduced in the absence of Flk2 (**Fig. 2D**), whereas B1a cells, ILC2s and MZBs were all significantly reduced in the absence of IL7Rα (**Fig. 2C, 2D, 2E**), consistent with prior studies of these cells in the same and other tissues, and peritoneal B1b and B2 cells (**Fig. S1B-B'**) (Hesslein, Yang and Schatz, 2006; Ghosn *et al.*, 2012; Patton *et al.*, 2014; Robinette *et al.*, 2017). Interestingly, despite being as efficiently labeled by both Flk2-Cre and IL7r-Cre (**Fig. 1H**), Tregs were not significantly reduced in either Flk2$^{-/-}$ or IL7Rα$^{-/-}$ mice, although there was a downward trend (**Fig. 2F**), as previously seen in thymic and splenic Tregs of IL7Rα$^{-/-}$ mice (Bayer *et al.*, 2008). We hypothesized that Flk2 may compensate for the lack of IL7Rα, and vice versa, allowing for near-normal Treg development in the absence of either receptor. To test this hypothesis, we generated **F**lk2, **I**L7Rα **d**ouble **k**nock**o**ut (FIDKO) mice and quantified total Tregs in the lung. We found a significant reduction of Tregs in the lung of FIDKO mice compared to WT mice (**Fig. 2F**), revealing an overlapping and cell-specific role of Flk2 and IL7Rα. We also quantified B1as, ILC2s and MZBs in the FIDKO mice and found that they recapitulated the severe reductions in numbers that we found in the IL7Rα$^{-/-}$ mice. These data suggest that both Flk2 and IL7Rα do have functional roles in TLC development during steady state hematopoiesis, although IL7Rα appears to be more important than Flk2 as indicated by the more severe reduction in cell numbers.

## Flk2$^{-/-}$ and IL7Rα$^{-/-}$ HSCs have impaired tissue-resident lymphoid cell reconstitution

Hematopoietic stem and progenitor cell (HSPC) differentiation relies on cytokine receptors such as Flk2 and IL7R to receive instructive signals from the environment (Zhang and Lodish, 2008). For example, we have previously shown that Flk2 is intrinsically required on HSPCs for both myeloid and lymphoid reconstitution, as demonstrated by the reduced capacity of Flk2$^{-/-}$ HSCs to generate myeloid and lymphoid progeny, including traditional circulating lymphoid cells, upon transplantation into a WT host (Beaudin, Boyer and Forsberg, 2014). More recently, we demonstrated an extrinsic requirement of IL7Rα in the generation of eosinophils from WT HSCs, as demonstrated by a reduced capacity of WT HSCs to generate eosinophils upon transplantation into an IL7Rα$^{-/-}$ host (Cool *et al.*, 2020). Therefore, we were curious if the deficiency in TLCs we observed in Flk2$^{-/-}$, IL7Rα$^{-/-}$ and FIDKO mice (**Fig. 2**) is due to the cell-intrinsic lack of receptors or due to changes to surrounding cells. To begin to answer this question, we transplanted WT, Flk2$^{-/-}$, IL7Rα$^{-/-}$ or FIDKO HSCs into sublethally irradiated wildtype hosts (**Fig. 3A**). Under such transplantation conditions, host cells are partially, but not completely, depleted (Boyer *et al.*, 2019), leading to robust and long-term HSC engraftment (**Fig. S3**). We first examined donor chimerism of circulating lymphoid cells. As expected, peripheral blood B cell donor chimerism was significantly lower from Flk2$^{-/-}$ donor HSCs, IL7Rα$^{-/-}$ HSCs and FIDKO HSCs compared to WT HSCs (**Fig. 3B**); similar results were observed for peripheral blood T cells (**Fig. S2B),** whereas GM reconstitution was significantly impaired only from Flk2$^{-/-}$ donor HSCs (**Fig. S3A**). When examining TLCs, we observed only donor chimerism of from IL7Rα$^{-/-}$ and FIDKO HSCs was significantly impaired, while Flk2 deficiency alone did not result in significant differences (**Fig. 3C-D, Fig. S1C,C'')**. A limitation of donor chimerism is that this measure relies on host cell numbers and therefore makes it more difficult to determine the intrinsic requirement of these receptors because the number of host cells change dynamically over time post-conditioning (Boyer *et al.*, 2019). To overcome this limitation, we quantified the absolute cellularity of donor-derived cells with a bead-based method that allows for simultaneous flow cytometric analysis and

counting, which is purely a measure of donor cells (Boyer *et al.*, 2019; Rajendiran, Boyer and Forsberg, 2020; Poscablo *et al.*, 2021). Quantification of absolute cellularity of donor-derived cells between WT and knockout HSCs revealed that reconstitution of B1a cells (**Fig. 3C'**) and MZBs (**Fig. 3D'**) was also significantly impaired by the loss of Flk2 alone. Regardless of quantification method, deletion of either IL7Rα alone, or both IL7Rα and Flk2, led to impaired reconstitution of all TLCs examined (**Fig. 3C'-F'**). This was intriguing since we did not observe significant reduction of Tregs in IL-7Rα$^{-/-}$ at steady state (**Fig. 2F**). This is likely due to the stress of transplantation which reveals more dynamic requirement of IL-7Rα, as we previously observed a similar trend when we compared granulocytes/macrophages at steady state in Flk2$^{-/-}$ mice to transplantation of Flk2$^{-/-}$ HSCs (Beaudin, Boyer and Forsberg, 2014). These data suggest that IL7Rα is required cell intrinsically for TLC reconstitution, that Flk2 cannot compensate for this requirement, and that IL7Rα is not capable of fully compensating for the loss of Flk2.

## WT HSCs have enhanced tissue-resident lymphoid cell reconstitution capacity in an IL7Rα$^{-/-}$ environment

Although the HSC transplantations of Figure 3 demonstrated a cell intrinsic requirement of IL7Rα for TLC reconstitution, it is also possible that extrinsic IL7Rα may be required, as we previously demonstrated for eosinophil reconstitution (Cool *et al.*, 2020). To test this, we performed transplantation of WT HSCs into IL7Rα-deficient mice (**Fig. 4A**). HSC engraftment in the bone marrow was similar between WT and IL7Rα$^{-/-}$ recipients (**Fig. S3B**), consistent with the lack of IL7R expression and function in HSCs. Surprisingly, we observed significantly greater numbers of donor-derived B1a cells, MZBs, ILC2s and Tregs (**Fig. 4B-E)**, as well as B1b and B2 cells (**Fig. S1D, D''**), but not circulating T (**Fig. S2C**) or myeloid cells (**Fig. S3C**), in the IL7Rα$^{-/-}$ hosts compared to WT hosts. This raised the question of whether the greater number of donor-derived cells in the IL7Rα$^{-/-}$ mice was reflective of an overall greater number of cells, or if the reconstitution by WT HSCs only compensated for the lower cell numbers of cells in the IL7Rα$^{-/-}$ host. Therefore, we determined the total number of host and donor derived cells

and compared these numbers between WT hosts and IL7Rα$^{-/-}$ hosts. We found no significant differences between WT and IL7Rα$^{-/-}$ recipients of total B1a (**Fig. 4B'**) and MZB (**Fig. 4C'**) cells, B1b and B2 cells (**Fig. S1D',D'''**) or peripheral blood T cells (**Fig. S2C'**). Interestingly, we observed significantly greater total numbers of ILC2s (**Fig. 4D'**) and Tregs (**Fig. 4E'**) in the IL7Rα$^{-/-}$ recipients compared to the WT hosts. Designation of each individual across experiments failed to reveal evidence of sex-specific trends or differences (**Fig. S4A-C'**), despite a previous report of differential KLRG1 receptor expression in ILC2s in males and females (Kadel *et al.*, 2018). Thus, the enhanced reconstitution of tissue-resident T lymphocytes in an IL7Rα$^{-/-}$ environment compared to consistent reconstitution of tissue-resident B lymphocytes suggests differential dependence on IL-7/IL7R signaling in the development of these cell types (Schluns *et al.*, 2000; Guimond *et al.*, 2009; Osborne *et al.*, 2011).

## WT HSCs transplanted into IL7Rα$^{-/-}$ mice are not fully capable of rescuing the impaired lymphoid phenotype

The majority of TLCs are thought to arise pre/perinatally, with little contribution from adult progenitors. Although we observed enhanced reconstitution of some TLCs by adult WT HSCs in IL7Rα$^{-/-}$ hosts, it remained unclear if the lymphoid deficiencies observed in the IL7Rα$^{-/-}$ mice (**Fig. 2**) were fully rescued by transplantation of adult WT HSCs. Therefore, we compared steady-state numbers of circulating lymphocytes and TLCs in both WT and IL7Rα$^{-/-}$ mice (**Fig. 2**, solid bars) to total numbers after long-term hematopoietic reconstitution post-transplantation of WT HSCs (**Fig. 4B'-4E')**. In WT and IL7Rα$^{-/-}$ mice transplanted with WT HSCs, we found no significant difference in total peripheral blood B cells or T cells, or peritoneal B1b and B2 cells, compared to WT mice at steady state (**Fig. 5A**, **Fig S2D**, **S1E-E'**). Similarly, we observed no difference between total numbers of MZBs in WT mice at steady-state and WT mice transplanted with WT HSCs (**Fig. 5C**, solid grey bar compared to grey lined bar). Therefore, WT HSCs were indeed capable of fully reconstituting these cells. In fact, although the difference in total MZBs was not significantly different between WT and IL7Rα$^{-/-}$ recipients (**Fig. 4C'**), we observed significantly more MZBs in the IL7Rα$^{-/-}$ recipient mice

compared to WT steady-state (**Fig. 5C**, solid grey bar compared to red lined bar). Interestingly, this was not the case for the other cell types examined. We observed significantly fewer B1a cells, ILC2s and Tregs in mice transplanted with WT HSCs compared to steady state WT mice (**Fig. 5B, 5D-E**; solid grey bars compared to grey and grey striped bars), consistent with reports by us and others of limited TLC potential of adult progenitors (Kikuchi and Kondo, 2006; Beaudin *et al.*, 2016; Schneider *et al.*, 2019) . Despite the enhanced reconstitution of ILC2 and Tregs observed in the IL7Rα$^{-/-}$ environment (**Fig. 4D'-E'**), we were surprised to find that this was not sufficient to return cell numbers to those observed in WT steady-state mice; rather, the total number of cells was not greater than the total numbers observed at steady-state in the IL7Rα$^{-/-}$ mice (**Fig. 5D-E**, solid red bars compared to red lined bars). Taken together, these data suggest that adult WT HSCs transplanted into IL7Rα$^{-/-}$ mice are not fully capable of rescuing the impaired lymphoid phenotype caused by IL7Rα deletion and that there is differential requirement for IL7Rα on the development of tissue-resident B and T cells.

## Fetal HSCs outcompete adult HSCs in TLC reconstitution

Our results in Figure 3 clearly demonstrate that adult HSCs can contribute robustly to all four subsets of TLCs that we investigated. As referenced above, there is evidence for a fetal origin of some TLCs (Mold *et al.*, 2010; Beaudin *et al.*, 2016; Schneider *et al.*, 2019; Azevedo Portilho *et al.*, 2021) and the relative contribution of fetal and adult HSCs to TLCs is unclear. There is particular controversy over the capacity of adult HSCs to contribute to B1a cells (Beaudin and Forsberg, 2016), as studies have reported opposing findings (E *et al.*, 2016; EE *et al.*, 2016; Kristiansen *et al.*, 2016; Sawai *et al.*, 2016). Therefore, we sought to determine the reconstitution capacity of both fetal and adult HSCs to generate TLCs. We performed competitive transplants of equal numbers of fetal and adult HSCs to allow for direct comparison of TLC reconstitution in the same host (**Fig. 6A**). We were surprised that fetal HSCs almost completely outcompeted adult HSCs, contributing significantly more to donor chimerism of B1a cells and MZBs (**Fig. 6B**) despite demonstrating reliable adult HSC reconstitution of B1a cells and MZB cells when transplanted without competing fetal HSCs (**Fig. 3C-D**). These findings were also observed for peritoneal B1b and B2 cells (**Fig. S5A)**, and peripheral blood GM, B and T

cells (**Fig. S5B)**. Interestingly, ILC2 and Treg donor chimerism was not as drastically different between fetal and adult HSC compared to B1a and MZB (**Fig. 6B**). To determine if the difference we observed in TLC reconstitution was due to HSC engraftment, we analyzed hematopoietic stem and progenitor cells (HSPC) in the bone marrow of recipient mice. We found no significant differences in donor chimerism of fetal derived HSCs compared to adult derived HSCs, or common myeloid progenitors (CMPs) (**Fig. 6C**). In contrast, we did observe significantly greater donor chimerism of fetal-derived common lymphoid progenitors (CLPs) compared to adult-derived CLPs. These data suggest that fetal reconstitution of B1a and MZB might rely heavily on CLP-dependent pathways of differentiation, while adult reconstitution of ILC2 and Tregs may differentiate via alternate, CLP-independent (but IL7Rα-dependent) pathways, as suggested previously (Ghaedi *et al.*, 2016).

# DISCUSSION

## Cell-intrinsic IL7Rα is required for differentiation of tissue-resident lymphoid cells.

In this study, we aimed to expand our understanding on the roles of the cytokine receptors Flk2 and IL7Rα beyond traditional, circulating lymphocytes by investigating their roles in TLC development and reconstitution. Using lineage tracing, knockout mouse models and transplantation assays, our data demonstrated a clear role of IL7Rα in the differentiation of tissue-resident lymphoid cells across multiple tissues. B1a cells, MZBs, ILC2 and Tregs all displayed very high labeling in both the FlkSwitch and Il7rSwitch lineage tracing models; however, only IL7Rα$^{-/-}$ resulted in severely impaired TLC development while the effects of Flk2$^{-/-}$ were minimal. Furthermore, IL7Rα$^{-/-}$ HSCs were incapable of reconstituting TLCs in WT hosts, while WT HSCs were capable of TLC reconstitution in IL7Rα$^{-/-}$ hosts, revealing a primarily cell-intrinsic requirement of IL7Rα for TLC differentiation. Interestingly, while adult HSCs were clearly capable of reconstituting all TLCs investigated, reconstitution of TLCs by fetal HSCs was notably greater compared to adult HSCs upon competitive transplantation.

## Flk2 is less essential for tissue-resident lymphocytes than circulating lymphocytes

Based on the high labeling in the FlkSwitch model (**Fig. 1**) and our previous finding that multipotent, myeloid and lymphoid progenitors, and PB B cells were reduced in Flk2$^{-/-}$ mice (Beaudin, Boyer and Forsberg, 2014), we expected to find significantly reduced numbers of TLCs in Flk2-deficient mice. We were surprised that only MZBs were significantly reduced in the Flk2$^{-/-}$ compared to WT mice (**Fig. 2, S2A**). We have also previously shown that multilineage reconstitution was impaired upon transplantation of Flk2$^{-/-}$ HSCs in WT recipients, likely due to the significant decrease in HSC differentiation into Flk2+ MPPs and CLPs (Beaudin, Boyer and Forsberg, 2014). Despite demonstrating that B1a cells, MZBs, ILC2 and Tregs differentiate via a Flk2 positive stage (**Fig. 1**), we were surprised that, amongst TLCs, only B1a and MZB reconstitution was impaired by the loss of Flk2 (**Fig. 3**). This may be due to a more stringent reliance on Flk2 for innate-like B cell rather than innate-like T cells, or reported CLP-independent differentiation of ILCs and T cells (Ghaedi *et al.*, 2016). The notion of partial CLP-independence for ILC2 and Treg differentiation is also supported by the fetal and adult HSC reconstitution data, as we observed no significant differences in fetal and adult contribution to ILC2s and Tregs (**Fig. 6B**) despite a drastic reduction in adult-derived CLPs (**Fig. 6C**). Interestingly, previous studies have shown that Tregs increase in number in response to Flt3 ligand in mice and humans (McGee, Edwan and Agrawal, 2010; Klein *et al.*, 2013), making it all the more surprising that Tregs are the least affected by the loss of Flk2. These data suggest that IL7Rα is able to compensate for the loss of Flk2 in a cell-type specific manner but not vice versa, and therefore plays a more essential role in TLC reconstitution.

## Absolute cell quantification and competitive HSC transplantation revealed unexpected reconstitution capability

In all transplantation experiments, we employed an absolute cell quantification method previously developed in the lab (Boyer *et al.*, 2019; Leung *et al.*, 2019; Cool *et al.*, 2020; Poscablo *et al.*, 2021). This method was critical to our interpretation of WT HSC

reconstitution capability in IL7Rα$^{-/-}$ recipients because it cannot be gleaned from percent donor chimerism alone. Since the IL7Rα$^{-/-}$ recipients were already devoid of any of the cells we were examining, donor-derived cell would be constituting 100% donor chimerism. By quantifying absolute cell numbers, we found that not only did WT HSCs generate TLCs in an IL7Rα$^{-/-}$ recipient, but that the overall numbers of ILC2 and Tregs were greater in the IL7Rα$^{-/-}$ recipients (**Fig. 4**). We suspect this is due to the abundance of IL-7 in the IL7Rα$^{-/-}$ mice (Martin *et al.*, 2017; Cool *et al.*, 2020) and lack of host competition for IL-7, which may in turn enhance the ILC2 and Treg reconstitution. Therefore, IL7Rα may have a cell non-autonomous role in regulating TLC subsets which rely more heavily on IL-7 signaling by controlling IL-7 levels (Martin *et al.*, 2017; Cool *et al.*, 2020). Additionally, in our previous examination of eosinophil reconstitution in IL7Rα$^{-/-}$ mice, we discovered that the concentrations of other cytokines involved in immune cell survival are different in IL7Rα$^{-/-}$ compared to WT mice (Cool *et al.*, 2020). Therefore, we speculate that differences in cytokine signals may play a significant role in the differences we observed in Figure 4. Testing of a number of candidates did not uncover significant upregulation of lymphoid-promoting factors, leaving the previously documented increase in IL7 levels the sole current candidate for the increased reconstitution in IL7Rα$^{-/-}$ mice over WT recipients.

We were also able to compare total TLCs reconstituted by WT HSCs to their total numbers at *in situ* at steady-state. With the exception of MZBs in the IL7Rα $^{-/-}$ host, WT HSCs in either the WT host or the IL7Rα $^{-/-}$ host did not fully rescue TLCs to WT steady state levels (**Fig. 5**). This could conceivably be because we performed these transplants with adult HSCs and at least some types of TLCs are thought to originate primarily from fetal/neonatal progenitors. Recent work from Locksley and colleagues reported that ILC2s from the lung originate from perinatal progenitors (Schneider *et al.*, 2019), and for decades it has remained controversial to what extent adult HSCs contribute to B1a cells. Here, we found robust B1a cell reconstitution from adult HSCs when transplanted alone (**Fig. 3B**), but this capacity was drastically overshadowed by fetal HSCs when transplanted competitively (**Fig. 6B**). Importantly, the fetal versus adult HSC competitiveness did not segregate by cell types often designated as fetal- versus adult-

derived, as fetal HSCs also contributed significantly more to other, adult-derived peritoneal and circulating immune cells (**Fig. S5**). Mechanistically, this was not due to differences in HSC engraftment (**Fig. 6C**), but more likely related to greater proliferative and lymphoid capacity of fetal relative to adult HSCs, also manifested by significant differences in CLP reconstitution and a trend towards more fetal-derived CMPs (**Fig. 6C**). These results could be explained by the presence of developmentally restricted HSCs (drHSCs) within the fetal, but not adult, HSC compartment (Beaudin *et al.*, 2016). While co-transplantation allowed us to more directly compare HSC reconstitution, our results show that competitive strategies may also obscure certain potential because one HSC type can outcompete the other in one or more lineages. This calls for close attention to the conditions of the assays used to claim reconstitution potential or lack thereof. The absolute quantification comparison may be a better indicator of the ability of adult HSCs to restore homeostasis, as these results revealed impairment in putative fetal-derived B1a, ILC2, and Tregs (**Fig. 5B, D, E**) but not adult-derived peritoneal or peripheral blood cells (**Fig. 5A, S1E-E', S2D**). These new perspectives will be valuable for more completely unraveling the cellular and molecular mechanisms that control TLC potential of fetal and adult HSCs; these clearly deserve further investigation, considering that HSCs are used in the clinic to treat a multitude of immune deficiencies.

## IL7Rα is required for both innate and adaptive immunity

Despite some TLCs displaying immune properties reminiscent of myeloid cells (Martin, Oliver and Kearney, 2001; Gunn and Brewer, 2006; Ha *et al.*, 2006; Drake and Kita, 2014; Panda and Colonna, 2019), we demonstrated here that they nevertheless rely on main regulators of traditional, circulating lymphoid cells (**Fig. 7**). The regulation of immune cell development by Flk2 and IL7R is highly complex, driven by partially overlapping expression and function of these receptors on hematopoietic progenitor cells (**Fig. 7A**) (Mackarehtschian *et al.*, 1995; Christensen and Weissman, 2001; Schlenner *et al.*, 2010; Boyer *et al.*, 2011). Additionally, we found that HSC competition can greatly influence the perceived ability to reconstitute hematopoiesis: when transplanted alone, adult HSCs provided robust TLC contribution (**Fig. 3**), which was almost completely overshadowed by fetal HSC-derived TLCs in competitive

transplantations (**Fig. 6**). This may lead to reassessment of “fetal-derived” cells and how to test and interpret the reconstitution potential of progenitor cells. Interestingly, IL7Rα regulation of TLC generation appeared predominantly cell intrinsic, similar to that of fetal-specified tissue-resident macrophages (Leung *et al.*, 2019), but in contrast to the solely cell extrinsic role we discovered for IL7Rα in adult myeloid eosinophil regulation (Cool *et al.*, 2020). The combination of cell intrinsic and extrinsic regulation by Flk2 and IL7R, and the dynamic secretion of their corresponding cytokines Flt3L and IL7 under different conditions support a model of cell interdependence and complex, tissue-specific feedback (**Fig. 7B**) (from Figures 1-5, S2-S3 and Beaudin, Boyer and Forsberg, 2014; Leung *et al.*, 2019; Cool *et al.*, 2020). This interconnectedness can be further unraveled by future tissue-specific deletions of Flk2, Flt3L, IL7Ra, IL7 and/or other cytokines that selectively promote immune development. Collectively, these data indicate broad and temporally dynamic functional roles of both Flk2 and IL7Rα beyond the typical lymphoid ascribed roles

# MATERIALS & METHODS

## Mice

All animals were housed and bred in the AAALAC accredited vivarium at UC Santa Cruz and group housed in ventilated cages on a standard 12:12 light cycle. All procedures were approved by the UCSC Institutional Animal Care and Use (IACUC) committees (OLAW assurance A3859-01; USDA Registration 93-R-0439, customer number 9198). Il7r-Cre (Schlenner *et al.*, 2010), and Flk2-Cre (Benz *et al.*, 2008) mice, obtained under fully executed Material Transfer Agreements, were crossed to homozygous Rosa26$^{mTmG}$ females (JAX Stock # 007576) (Muzumdar *et al.*, 2007) to generate "switch" lines, all on the C57BL/6 background. WT C57BL/6, Rosa26$^{mTmG}$, UBC-GFP (JAX Stock # 004353) (Schaefer *et al.*, 2001) and KuO (Hamanaka *et al.*, 2013) mice were used for controls. FIDKO (**F**lk2 **I**l7rα **D**ouble **K**nock-**O**ut) mice were generated by crossing Flk2$^{-/-}$ (Mackarehtschian *et al.*, 1995) and IL7Rα $^{-/-}$ (Peschon *et*

*al.*, 1994) mice to homozygosity. Adult male and female mice (8-12 weeks old) were used randomly and indiscriminately, with the exception of the FlkSwitch line, in which only males were used because many female mice do not carry a Cre allele.

## Tissue and cell isolation

Mice were sacrificed by $CO_2$ inhalation. Adult lung and spleen were isolated, weighed, then placed into 1.5mL of digestion buffer (1x PBS(+/+) with 2% serum, 1 mg/mL (lung) or 2mg/ml (spleen) collagenase IV (Gibco) with 100U/ml DNase1) containing Calibrite APC-labeled (BD Biosciences) counting beads and manually disassociated into 2mm x 2mm pieces using surgical scissors. The tissues were then incubated at 37°C for 1 hour (lung) or 2 hours (spleen). Following incubation, all tissues were passaged through 19g then a 16g needle 10 times, and then filtered through a 70 µM filter and 25mL of cell staining buffer (1X PBS with 2% serum and 5mM EDTA) was added to quench the digestion enzymes.

## Flow Cytometry & Cell Analysis

Cell labeling was performed on ice in 1X PBS with 5 mM EDTA and 2% serum. Analysis was performed on a BD FACS Aria III (BD Bioscience, San Jose, CA) at University of California-Santa Cruz, and analyzed using FlowJo (Treestar)(Boyer *et al.*, 2019; Smith-Berdan *et al.*, 2019; Martin *et al.*, 2021; Poscablo *et al.*, 2021). Cells were defined as follows: B1a = $Lin^-$ (Ter119, CD11b, CD3) $IgM^+$ $CD5^+$ $CD11b^{mid}$; MZB = $Lin^-$ (CD3, CD4, CD8, Ter-119, Gr1) $B220^+$ $IgM^+$ $CD21^+$ $CD23^-$; ILC2 = $Lin^-$ (CD3, CD4, CD5, CD8, NK1-1, CD19, Ter119, F4/80, FcεRIα) $CD45^+$ $KLRG1^+$ $Sca\text{-}1^+$ $CD25^+$; Treg = $Lin^-$ (Ter119, Gr1) $CD3^+$ $CD4^+$ $TCRB^+$ $CD25^+$ $FOX\text{-}P3^+$.

## Transplantation Assays

Transplantation assays were performed as previously described (Beaudin, Boyer and Forsberg, 2014; Smith-Berdan *et al.*, 2015; Ugarte *et al.*, 2015; Beaudin *et al.*, 2016). Briefly, double sorted HSCs were isolated from bone marrow of either WT (non-fluorescent, mTmG, UBC-GFP or KuO), $Flk2^{-/-}$, $Il7R\alpha^{-/-}$ or FIDKO 8-12 week old mice

and from E14.5 fetal livers. HSCs were defined as KLS (cKit$^{+}$Lin$^{-}$Sca-1$^{+}$), CD150$^{hi}$Flk2$^{-}$. Lineage markers were CD3, CD4, CD5, CD8, B220, Mac-1, Gr-1 and Ter-119. Mac1 antibodies were excluded from the lineage cocktail when sorting fetal progenitors (Morrison *et al.*, 1995). Flk2 could not be used to identify HSCs in Flk2$^{-/-}$ and FIDKO HSCs and so they were sorted on KLS, CD150$^{hi}$. Recipient mice aged 8-12 weeks were sublethally irradiated (750 rad, single dose) with a Faxitron CP-160 (Faxitron). Under isofluorane-induced general anesthesia, sorted cells were transplanted retro-orbitally. Recipient mice were bled 4, 8, 12, and 16 weeks post transplantation via tail vein and peripheral blood (PB) was analyzed for donor chimerism by means of fluorescence profiles and antibodies to lineage markers (**Table 1**). Long-term multilineage reconstitution was defined as chimerism in both the lymphoid and myeloid lineages of > 0.1% at 16 weeks post-transplantation and only mice that displayed long-term reconstitution were used for post-transplantation analysis.

## Absolute Cell Number Quantification

A known volume of PB was mixed with an antibody solution, (PBS, 5mM EDTA and 2% serum) containing a known quantity of Calibrite APC beads prior to flow cytometry analysis (Boyer *et al.*, 2019; Cool *et al.*, 2020; Rajendiran, Boyer and Forsberg, 2020; Poscablo *et al.*, 2021). For tissues, a known quantity of beads was added to each at the very beginning of tissue preparation prior to antibody staining and analysis. The number of beads counted by flow cytometry was used to calculate the number of mature cells per micro liter of blood or within each tissue.

## Quantification and Statistical Analysis

Number of experiments, n, and what n represents can be found in the legend for each figure. Power analysis to determine the minimum size of experimental groups were performed with a confidence level of 0.95 and desired power of 0.8 using an online tool (Assessment, 2009). Statistical significance was determined by two-tailed unpaired student's T-test when comparing two groups and by one-way ANOVA and Dunnett

multi-parameter test when comparing across multiple groups. In figures 2-4, 6, S2A-C', S3-S5, all comparisons are being made to WT controls, and not across all groups despite being graphed together. All data are shown as mean ± standard error of the mean (SEM) representing at least two independent experiments.

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## COMPETING INTERESTS

The authors have declared that no competing interests exist.

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# Figures

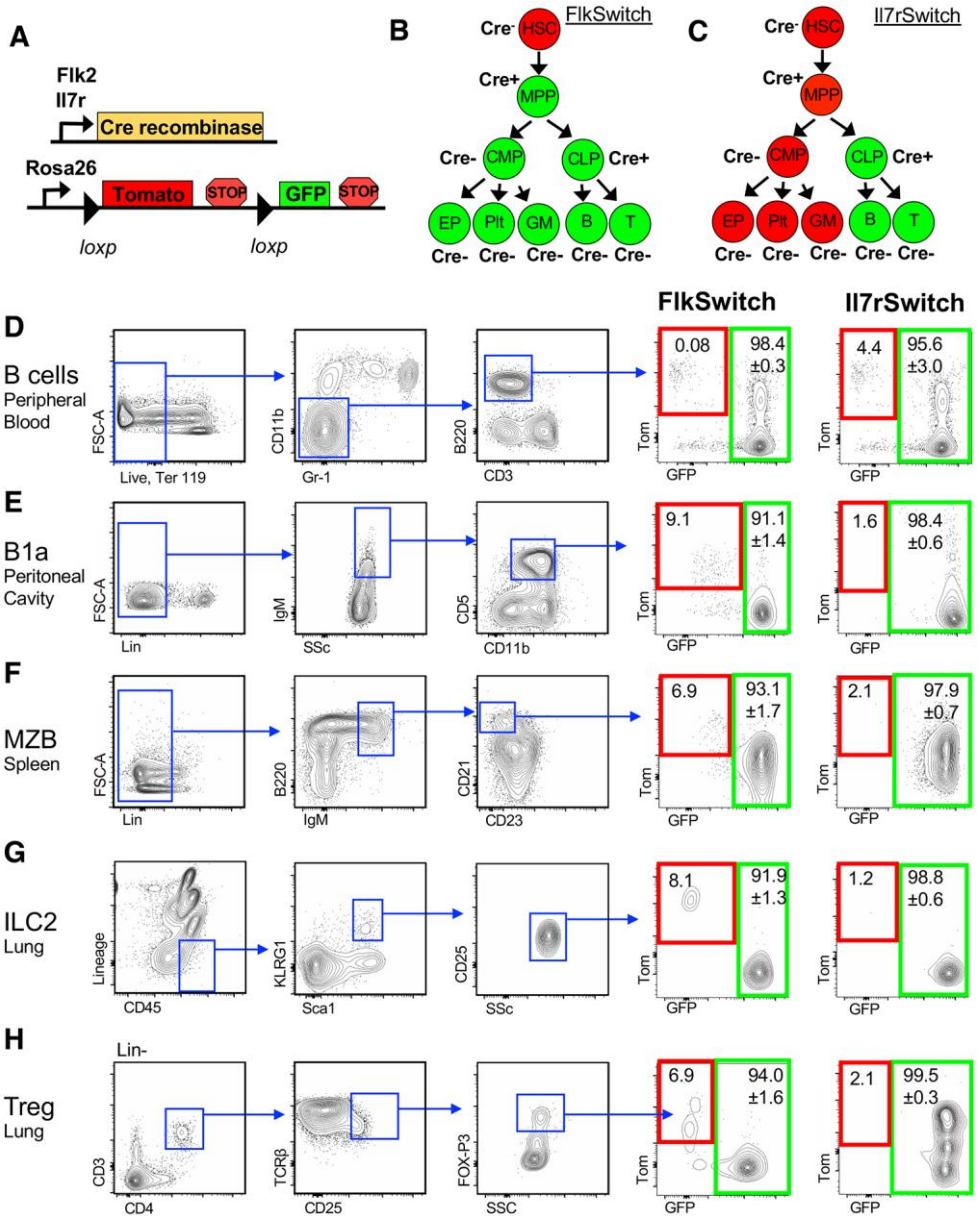


**Figure 1: Flk2-Cre & IL7R-Cre efficiently labeled tissue-resident lymphoid populations.**

**A**) Genetics of the “Switch" models. Flk2 or IL7R regulatory elements drive Cre recombinase expression. Flk2-Cre or IL7r-Cre mice were crossed to Rosa26$^{mTmG}$ mice expressing a dual color reporter expressing either Tomato (Tom) or GFP.

**B**,**C)** Schematic of Cre-mediated reporter switching in the “FlkSwitch" (B) and “IL7rSwitch” (C) models. Expression of Cre results in an irreversible switch from Tomato to GFP expression. Once a cell expresses GFP, it can only give rise to GFP-expressing progeny. These models represent Cre-driven labeling in young adult steady state hematopoiesis of circulating, “traditional”, mature myeloid and lymphoid cells.

**D-H)** TLCs were highly labeled in both FlkSwitch and IL7rSwitch lineage tracing models. Representative flow cytometric analysis of reporter expression across “traditional” circulating B cells and TLCs, all pre-gated on singlets, lymphocytes and live cells.

**D)** “Traditional” B cells (Ter 119$^{-}$ CD11b$^{-}$ Gr-1$^{-}$ CD3$^{-}$ B220$^{+}$) in the peripheral blood and different tissue-resident lymphoid populations in adult mice:

**E)** B1a (Lin$^{-}$IgM$^{+}$CD5$^{+}$CD11b$^{mid}$) cells in the peritoneal cavity;

**F**) MZB: Marginal Zone B cells (Lin$^{-}$B220$^{+}$IgM$^{+}$CD21$^{+}$CD23$^{-}$) in the spleen;

**G)** ILC2: Innate Lymphoid cell Type 2 (Lin$^{-}$CD45$^{+}$KLRG$^{+}$Sca-1$^{+}$CD25$^{+}$) in the lung; and

**H)** Tregs: regulatory T cells (Lin$^{-}$CD3$^{+}$CD4$^{+}$TCRB$^{+}$CD25$^{+}$FOX-P3$^{+}$) in the lung. Tom and GFP expression are highlighted by red and green boxes, respectively, in FlkSwitch and IL7RSwitch models. Values indicate mean frequencies ± SEM of gated Flk2-Cre and IL7R-Cre marked GFP+ populations. Data are representative of 4-5 mice per cohort in three independent experiments.

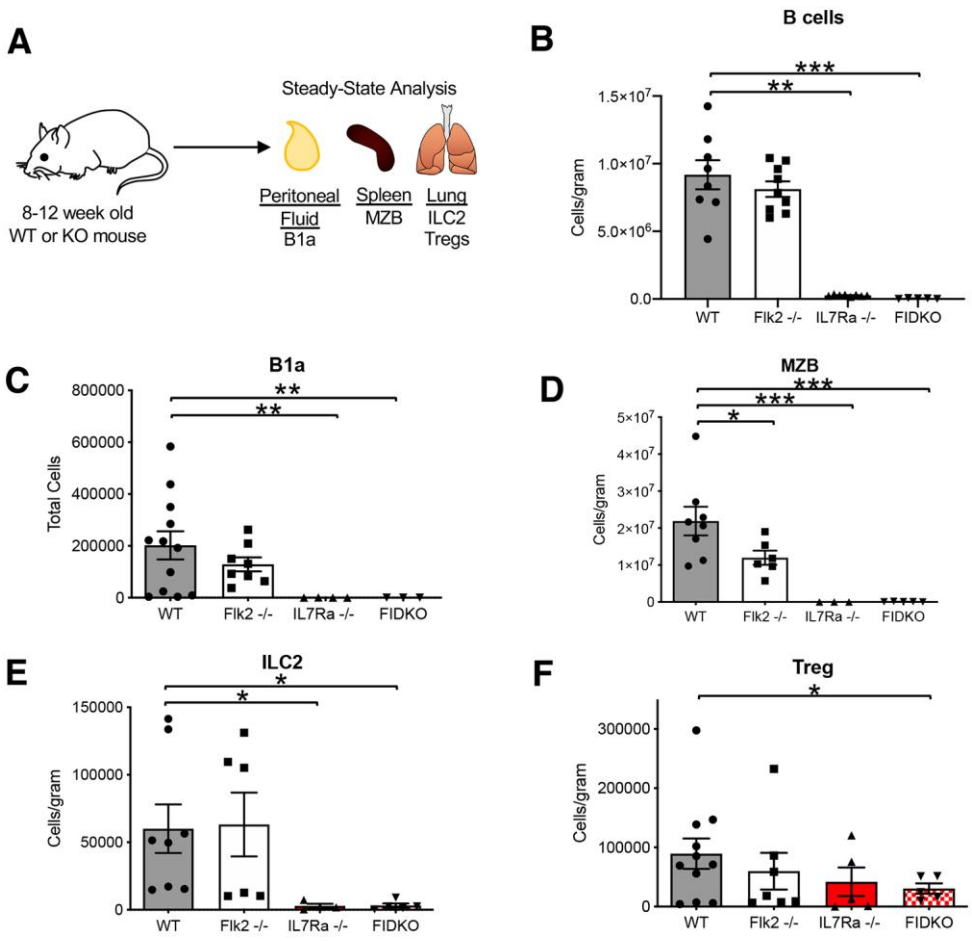



**Figure 2: Tissue-resident lymphoid cells are severely reduced in the absence of IL7Rα, but not Flk2.**

**A)** Schematic of experimental design. Peritoneal fluid, spleen and lung from 8 to 12 week old WT, Flk2$^{-/-}$, IL7Rα$^{-/-}$ and FIDKO HSCs were harvested and analyzed for cellularity of TLCs and represented in bar plots of. WT (grey), Flk2$^{-/-}$ (white), IL7Rα$^{-/-}$ (red) and Flk2$^{-/-}$/IL7Rα$^{-/-}$ double knockout (FIDKO; red/white).

**B)** "Traditional" B cells in the lung were significantly reduced in both IL7Rα$^{-/-}$ and FIDKO mice, but not in Flk2$^{-/-}$ mice. Quantification of B cells (Live, Ter119- Mac1- Gr1- CD19+) per gram of tissue in the lungs.

**C)** B1a cells in the peritoneal cavity were significantly reduced in IL7Rα$^{-/-}$ and FIDKO mice compared to WT. Quantification of total cell numbers in the peritoneal cavity.

**D)** MZBs were significantly reduced in Flk2$^{-/-}$, IL7Rα$^{-/-}$ and FIDKO compared to WT mice. Quantification of cells/gram of tissue in the spleen.

**E)** ILC2s were significantly reduced in IL7Rα$^{-/-}$ and FIDKO compared to WT mice. Quantification of cells/gram of tissue in the lung.

**F)** Tregs were significantly reduced only in FIDKO compared to WT mice. Quantification of cells/gram of tissue in the lung. WT n=8 (all male), Flk2$^{-/-}$ n=6 (all male), IL7Rα$^{-/-}$ n=4 (all male), FIDKO n=5 (3 male); representing four independent experiments, mean +/- S.E.M. Differences were analyzed with two-tailed Student t test *, P<0.05; **, P< 0.005; ***, P< 0.0005

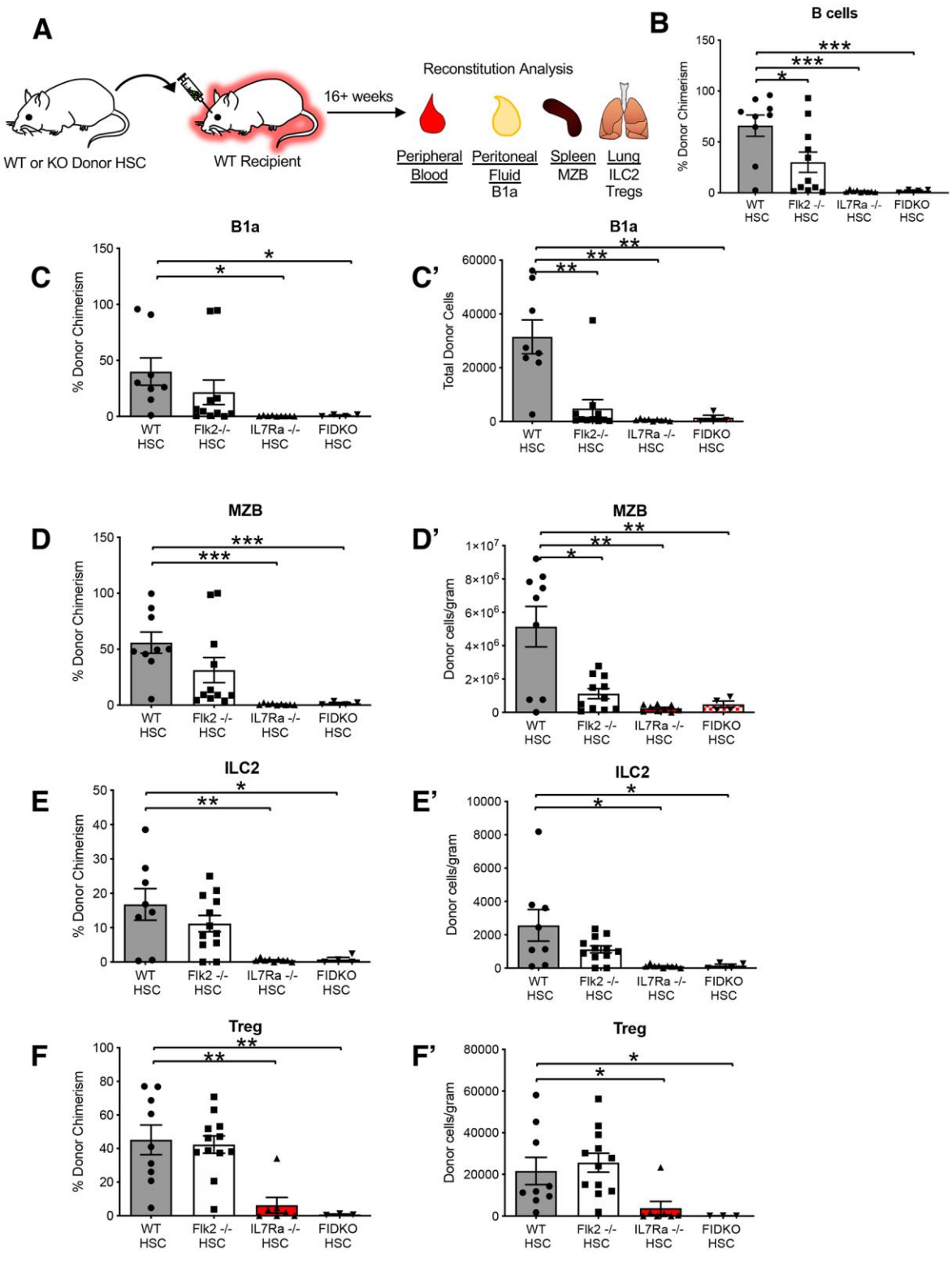


**Figure 3: IL7Rα$^{-/-}$ and Flk2$^{-/-}$ HSCs had impaired tissue-resident lymphoid cell reconstitution compared to WT HSCs.**

**A)** Schematic of experimental design. 500 WT, Flk2$^{-/-}$, IL7Rα$^{-/-}$ or FIDKO HSCs were transplanted into sublethally irradiated fluorescent WT recipients (mTmG or UBC-GFP) and donor contribution of TLCs was quantified 16 weeks post transplantation.

**B)** Reconstitution of peripheral blood B cells was significantly impaired upon transplantation of Flk2$^{-/-}$(white bars), IL7Rα$^{-/-}$ (red bars), or FIDKO HSCs (red/white bars) compared to WT HSCs (grey bars). Quantification of donor chimerism of "traditional" B cells in the peripheral blood.

**C-F)** Donor chimerism of TLCs significantly reduced from IL7Rα$^{-/-}$ (red bars) and FIDKO (red square bars) donor HSCs compared to WT (grey bars) donor HSCs. Quantification of donor chimerism of B1a cells, MZBs, ILC2s and Tregs, respectively.

**C'-F')** Cellularity of B1a and MZB cells were significantly reduced from Flk2$^{-/-}$ (white bars), IL7Rα$^{-/-}$ (red bars) and FIDKO (red square bars) donor HSCs compared to WT (grey bars) donor HSCs. Cellularity of lung ILC2 and Tregs were significantly reduced from, IL7Rα$^{-/-}$ (red bars) and FIDKO (red square bars), but not Flk2$^{-/-}$ (white bars), donor HSCs compared to WT (grey bars) donor HSCs. Quantification of total B1a cells, cells/gram spleen of MZBs, cells/gram lung ILC2s and cells/gram of lung tissue Tregs, respectively. WT n=8 (6 male), Flk2$^{-/-}$ n=12 (8 male), IL7Rα$^{-/-}$ n=7 (4 male), FIDKO n=4 (3 male), representing a minimum of four independent experiments, mean +/- S.E.M. Differences were analyzed with two-tailed Student t test *, $P<0.05$; **, $P< 0.005$; ***, $P<0.0005$.

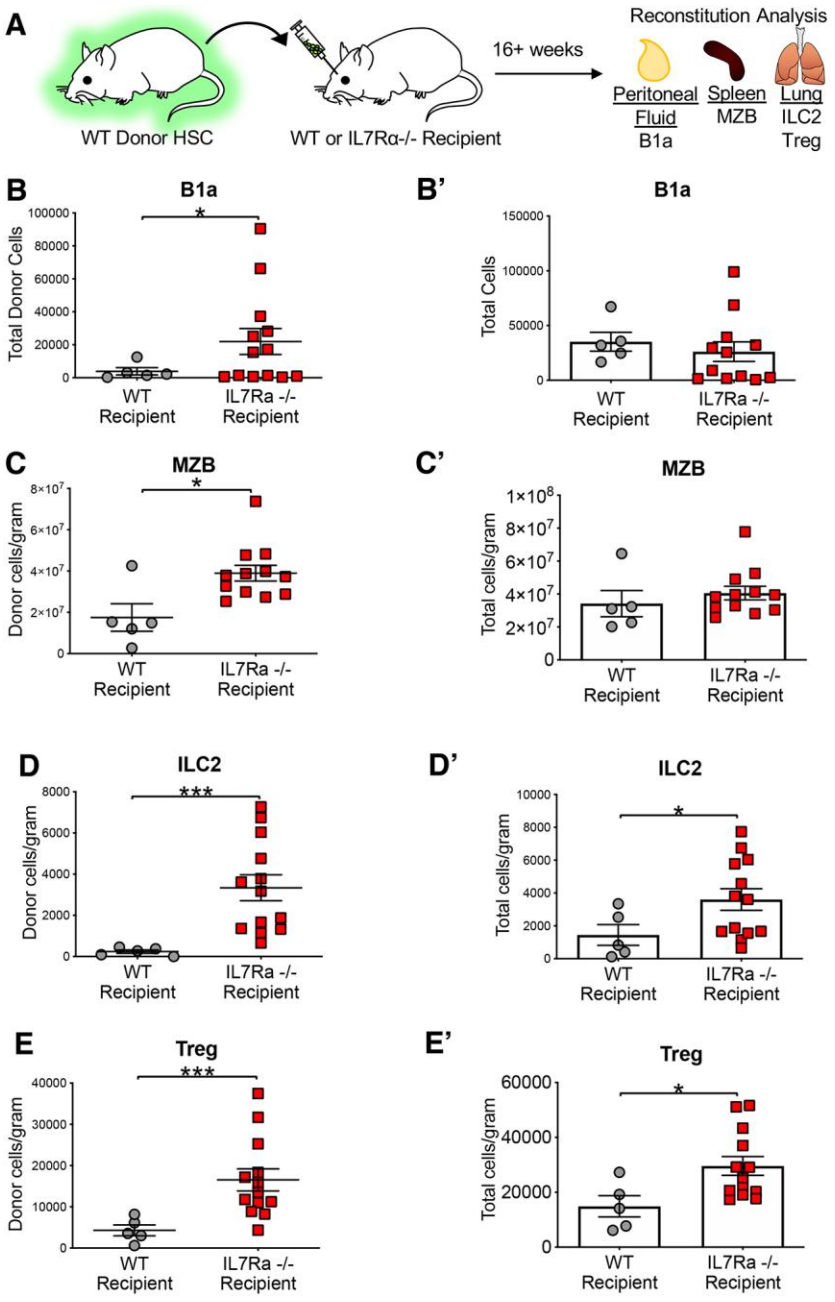



**Figure 4: WT HSCs have enhanced non-traditional lymphoid cell reconstitution capacity in an IL7Rα$^{-/-}$ environment.**

**A)** Schematic of experimental design. 500 fluorescent WT HSCs (UBC-GFP), were transplanted into sublethally irradiated non-fluorescent WT or IL7Rα$^{-/-}$ recipients. Donor contribution to TLCs was quantified 16 weeks post-transplantation.

**B-E)** Significantly greater donor-derived TLCs in an IL7Rα$^{-/-}$ recipient (red squared) compared to a WT recipient (grey dots). Quantification of donor-derived cells of B1a cells, ILC2s, MZBs and Tregs, respectively.
**B'-E')** Significantly greater total ILC2 and Treg cells in an IL7Rα$^{-/-}$ recipient (red squared) compared to a WT recipient (grey dots). Quantification of total cells (donor+host) of B1a cells, ILC2s, MZBs and Tregs, respectively. WT recipient n=5 (1 male), IL7Rα$^{-/-}$ recipient n=13 (2 male), representing four independent experiments, mean +/- S.E.M. Differences were analyzed with two-tailed Student t test *, P<0.05; **, P< 0.005; ***, P< 0.0005.

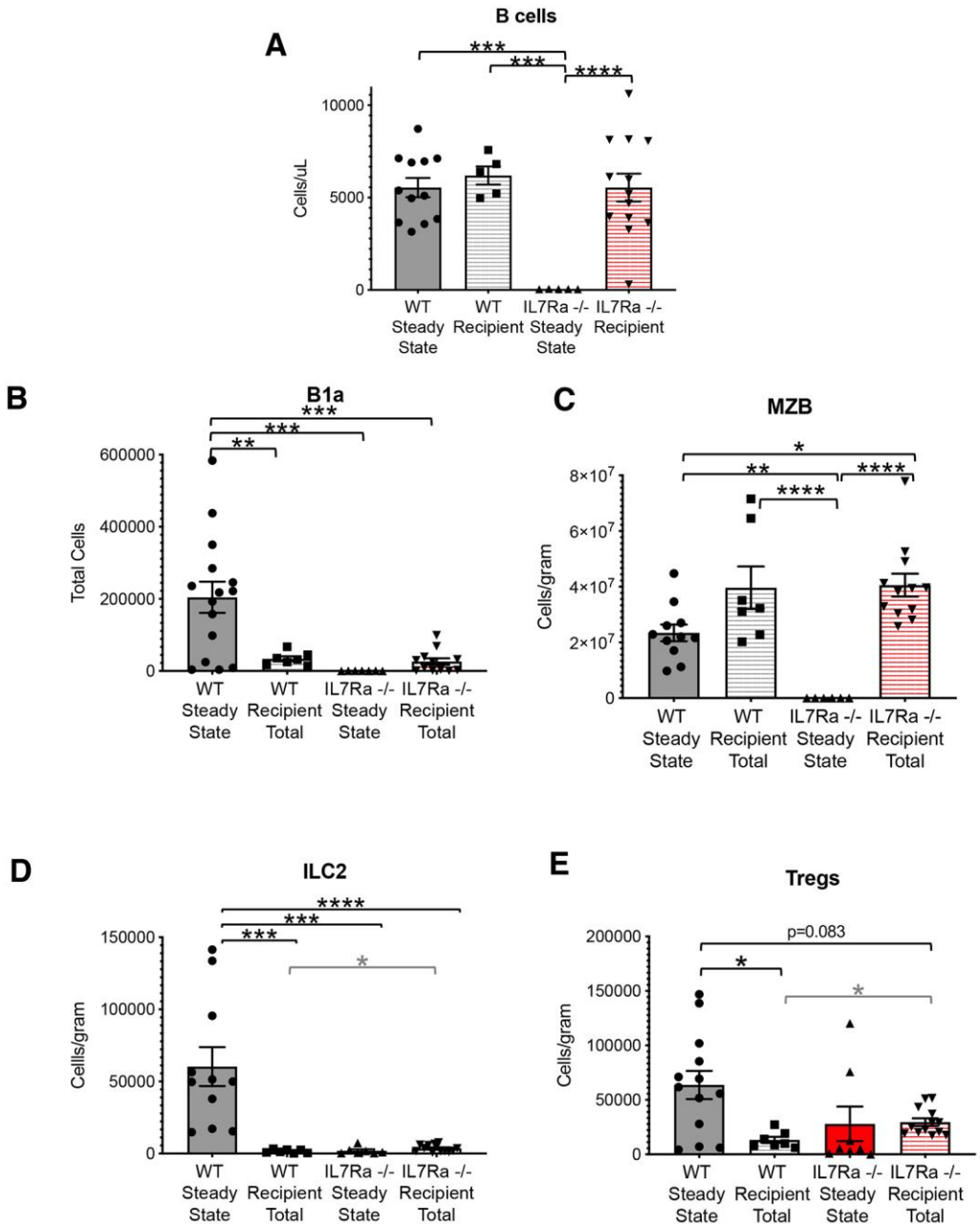



**Figure 5: WT HSCs transplanted into IL7Rα$^{-/-}$ mice are not capable of reconstituting tissue-resident lymphocytes to steady state numbers (or levels).** Bar graph comparing circulating B cells and TLCs cells at steady state (solid bars) to their reconstitution upon transplantation of WT HSCs (pattern bars).

**A)** Cellularity of peripheral blood B cells,

**B)** B1a cells,

**C)** MZB,

**D)** ILC2 and

**E)** Tregs in WT or IL7Rα$^{-/-}$ mice are compared to WT and IL7Rα$^{-/-}$ mice reconstituted with WT HSCs. WT Steady state n=11; WT Recipient Total n=7; IL7Rα$^{-/-}$ steady state n=6; IL7Rα$^{-/-}$ Recipient Total n=12. Differences were analyzed with One-way ANOVA; PB B cells $p<0.0001$; B1a cells $p<0.0001$; MZB $p<0.0001$; ILC2 $p<0.0001$; Tregs = 0.0134. Dunnett multi-parameter test *, $P<0.05$; **, $P< 0.005$; ***, $P< 0.0005$; ****, $P< 0.00005$. In gray are differences analyzed with two-tailed Student t test as reported in Figure 4, *, $P<0.05$.

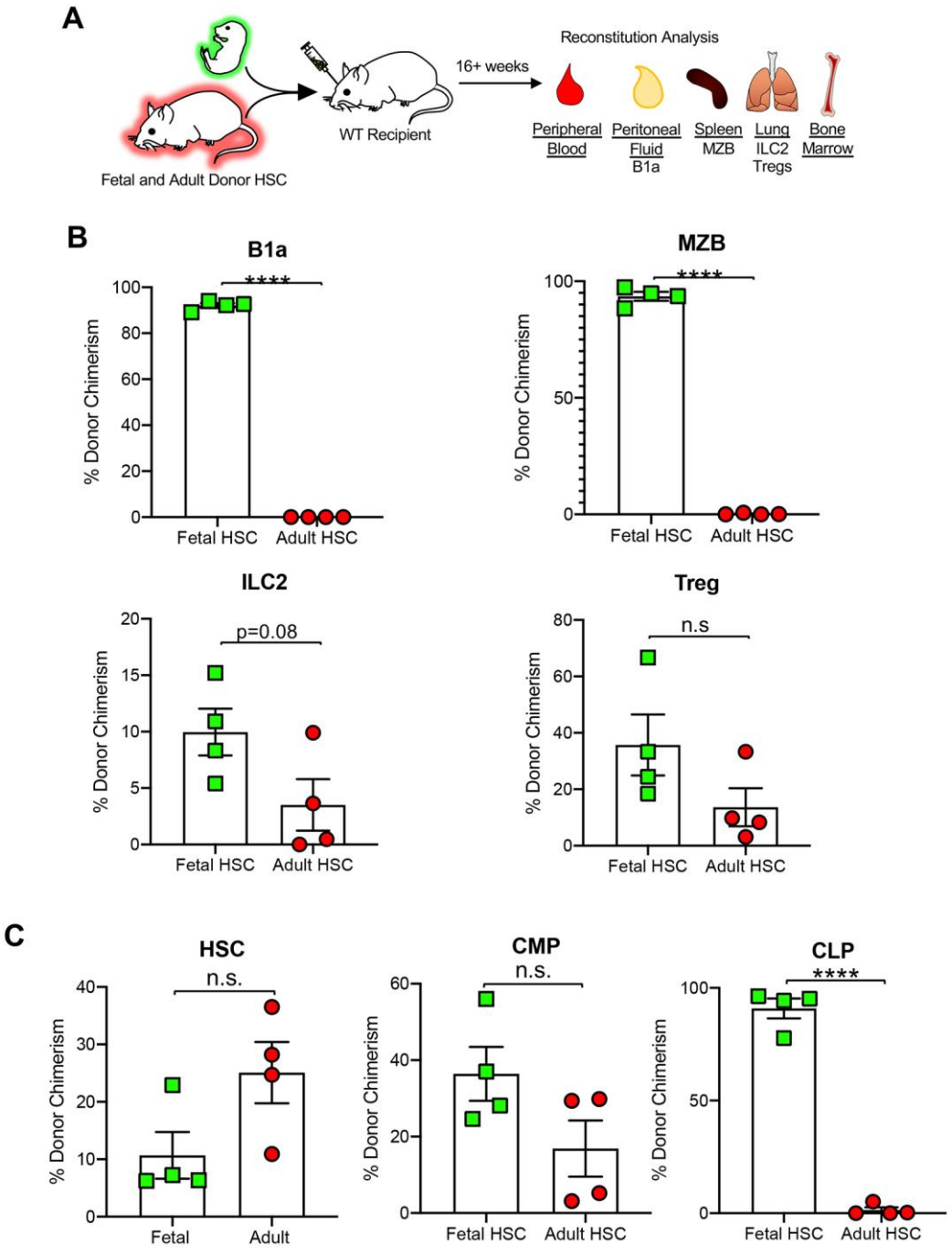



**Figure 6: Fetal HSCs reconstitute TLCs with greater efficiency compared to adult HSCs.**

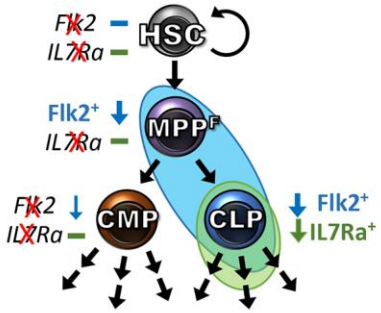
**A)** Schematic of experimental design. 250 fetal HSCs and 250 adult HSCs from different fluorescent mice (KuO and UBC-GFP, respectively) were co-transplanted into

sublethally irradiated WT recipients and donor contribution to hematopoietic cells was quantified in several organs 16 weeks post transplantation.

**B)** Donor chimerism of TLCs was significantly greater by fetal HSCs compared to adult HSCs. Quantification of donor chimerism of peritoneal B1a cells, splenic MZBs, and lung ILC2s and Tregs.

**C)** Fetal and adult HSCs engrafted in the BM with similar efficiencies, but donor chimerism of fetal-derived CLPs was significantly greater than adult-derived CLPs. Quantification of donor chimerism of fetal and adult HSC, CMP and CLP in recipient BM. Fetal HSC n=4, Adult HSC n=4, two independent experiments. Differences were analyzed with two-tailed Student t test *, $P<0.05$; **, $P< 0.005$; ***, $P<0.0005$.

A




B

| | Cell type | Lineage tracing | | Steady-state | | Reconstitution | | Recipient |
|---|---|---|---|---|---|---|---|---|
| | | FlkSwitch | IL7RαSwitch | Flk2$^{-/-}$ | IL7Rα$^{-/-}$ | Flk2$^{-/-}$ | IL7Rα$^{-/-}$ | IL7Rα$^{-/-}$ |
| Traditional | B cells | | | ↓ | ↓ | ↓ | ↓ | – |
| | myeloid | | | – | – | ↓ | – | – |
| | T cells | | | ↓ | ↓ | ↓ | ↓ | – |
| Tissue-resident | trMacs | | | – | ↓ | *ND* | *ND* | *ND* |
| | eosinophils | | | ↓ | ↓ | – | – | ↓ |
| | TLCs | | | ↓ | ↓ | ↓ | ↓ | ↑ |

**Figure 7: Model summary of Flk2- and IL7R-mediated effects on hematopoiesis.**

**A)** The Flk2 and IL7R effects on downstream progeny are likely asserted at least in part by their expression on early hematopoietic progenitors, including multipotent progenitor cells (MPP$^{F}$) and common lymphoid progenitors (CLPs). Flk2 expression on MPPs, CLPs, and downstream lymphoid cells is indicated by blue Flk2+ text and by blue background. IL7R expression on CLPs and downstream lymphoid cells is indicated by green IL7R+ text and green background. The blue and green arrows indicate that the respective cell type is reduced upon deletion of either Flk2 or IL7R.

**B)** Differential effects of Flk2 and IL7R in the development and maintenance of hematopoietic cell types. Columns depict cell types, lineage tracing, steady-state analysis, HSC reconstitution of wild-type recipients, and WT HSC transplantation into IL7R-deficient recipients. Rows depict six categories of immune cells: “traditional” B, T, or myeloid cells (rows 1-3), or tissue-resident macrophages (trMacs), lung eosinophils, and lymphoid cells (TLCs) (rows 4-6). Results of Flk2 and IL7R lineage tracing are

indicated in red (no history of expression) and green (differentiation occurred via cells expressing Flk2 or IL7R). Results of Flk2 and IL7R deletion on cell numbers is indicated by arrows pointing up (increase) or down (decrease); no change is indicated by a horizontal bar; N.D. = no data.

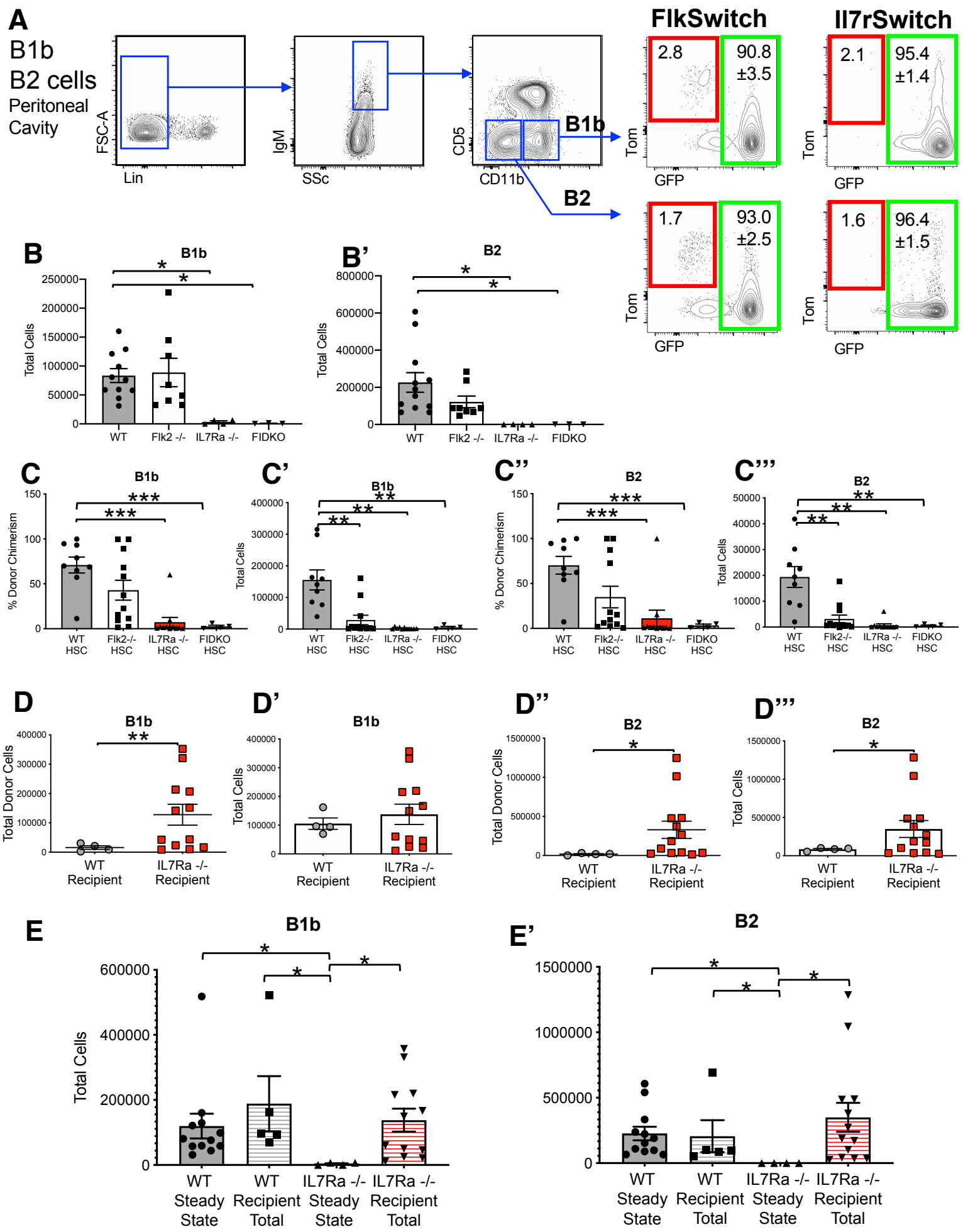


**Fig. S1. B1b and B2 cells from the peritoneal cavity behave like traditional, circulating B-cells.**

**A)** B1b ($Lin^-IgM^+CD5^-CD11b^{mid}$) and B2 ($Lin^-IgM^+CD5^-CD11b^-$) cells from the peritoneal cavity were highly labeled in both FlkSwitch and IL7rSwitch lineage tracing models. Representative flow cytometric analysis of reporter expression.

**B)** B1b and **B')** B2 cells were significantly reduced in IL7Rα$^{-/-}$ and FIDKO mice compared to WT. Quantification of total cell numbers in the peritoneal cavity. Data are representative of 4-5 mice per cohort in three independent experiments.

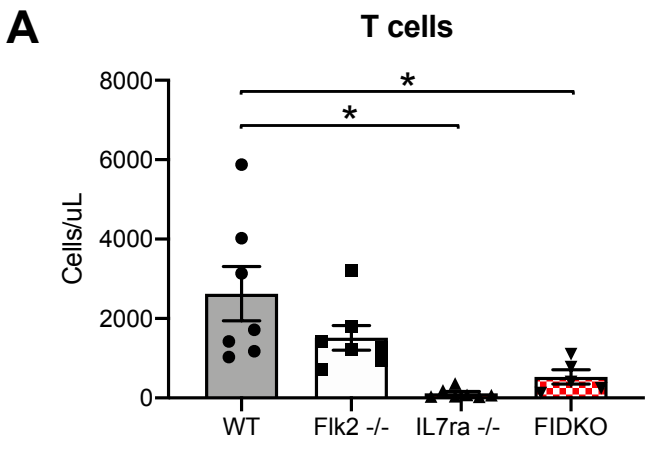
**C, C'')** Donor Chimerism of B1b and B2 cells was significantly reduced from IL7Rα$^{-/-}$ (red bars) and FIDKO (red square bars) donor HSCs compared to WT (grey bars) donor HSCs. Quantification of donor chimerism of B1b and B2 cells respectively.

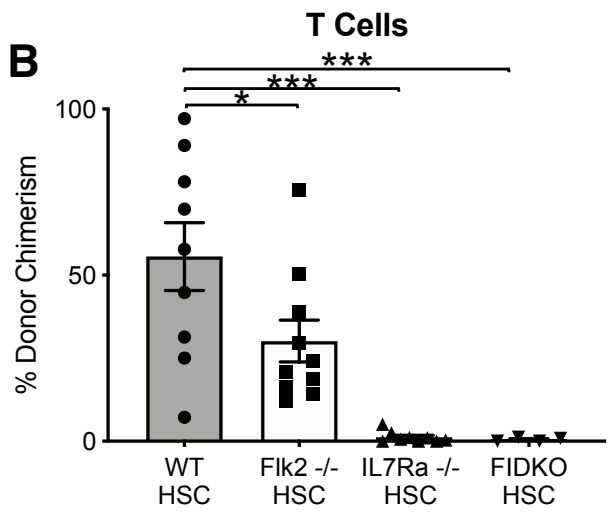
**C', C''')** Cellularity of B1b and B2 cells was significantly reduced from Flk2$^{-/-}$ (white bars), IL7Rα$^{-/-}$ (red bars) and FIDKO (red square bars) donor HSCs compared to WT (grey bars) donor HSCs. Quantification of total B1b and B2 cells. WT n=9, Flk2$^{-/-}$ n=11, IL7Rα$^{-/-}$ n=7, FIDKO n=4, representing a minimum of four independent experiments. **D, D'')** Significantly greater donor-derived B1b and B2 cells in an IL7Rα$^{-/-}$ recipient (red squares) compared to a WT recipient (grey dots). Quantification of donor-derived cells of B1b and B2 cells, respectively.

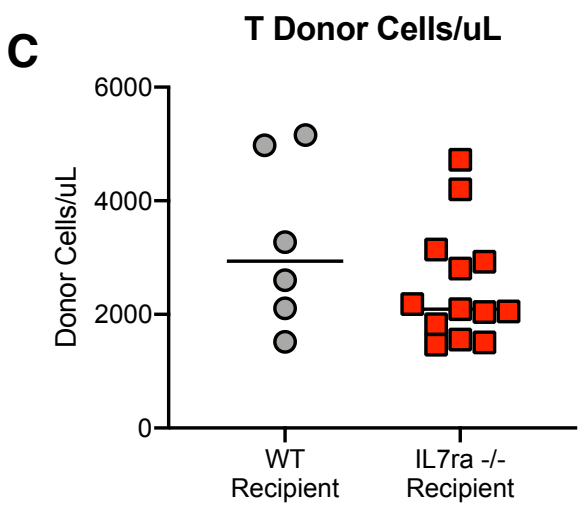
**D', D''')** Significantly greater total B2 cells in an IL7Rα$^{-/-}$ recipient (red squared) compared to a WT recipient (grey dots). Quantification of total cells (donor+host) of B1b and B2, respectively. WT recipient n=5, IL7Rα$^{-/-}$ recipient n=13, representing four independent experiments.

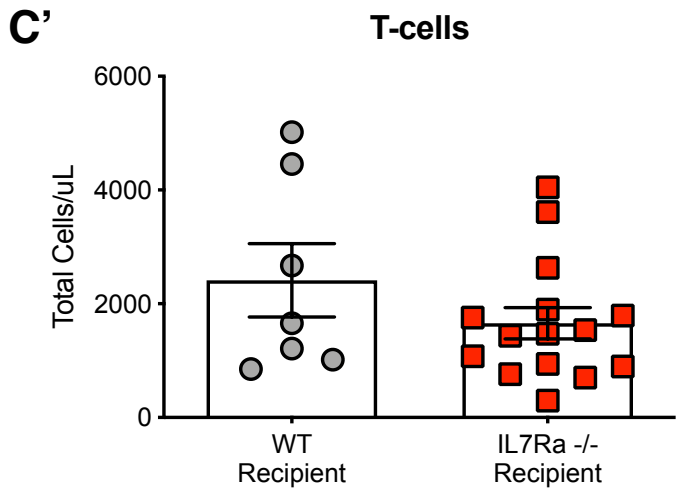
**E-E')** Bar graph comparing circulating B cells and TLCs cells at steady state (solid bars) to their reconstitution upon transplantation of WT HSCs (pattern bars). Cellularity of B1b and B2 in WT or IL7Rα$^{-/-}$ mice are compared to WT and IL7Rα$^{-/-}$ mice reconstituted with WT HSCs.

Differences in B-D) were analyzed with two-tailed Student t test; *, $P<0.05$; **, $P< 0.005$; ***, $P<0.0005$. Differences in E-E') were analyzed with one-way ANOVA; B1b p=0.0107 and B2 p=0.0129 and Dunnett multi-parameter test; *, $P<0.05$; **, $P< 0.005$; ***, $P<0.0005$.



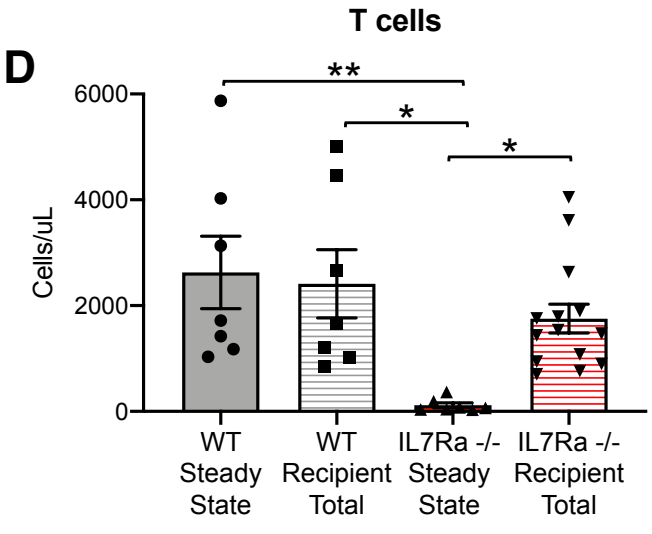



**Fig. S2. CD3+ T cells in peripheral blood require IL7Rα.**

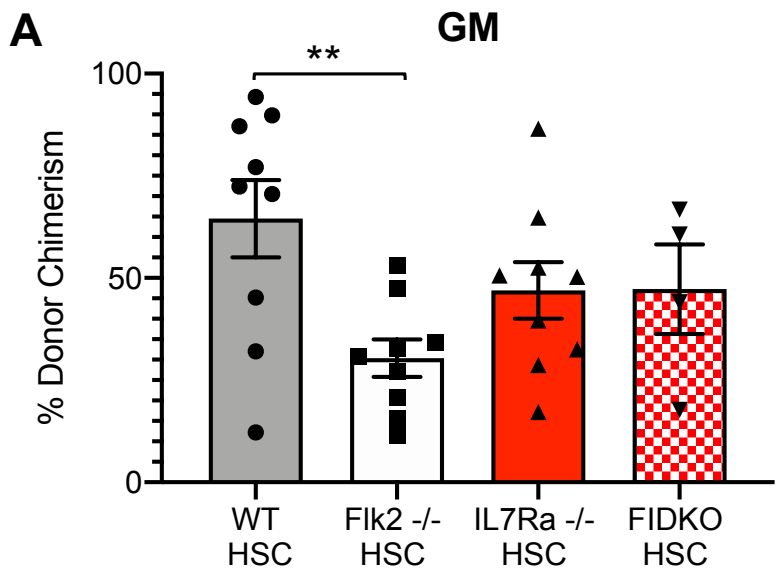
**A)** Numbers of "traditional" CD3+ T cells in the peripheral blood of 8 to 12 week old WT, Flk2$^{-/-}$, IL7Rα$^{-/-}$ and FIDKO mice. WT (grey), Flk2$^{-/-}$ (white), IL7Rα$^{-/-}$ (red) and Flk2$^{-/-}$ /IL7Rα$^{-/-}$ double knockout (FIDKO; red/white). Data from the same mice as in Figure 2.

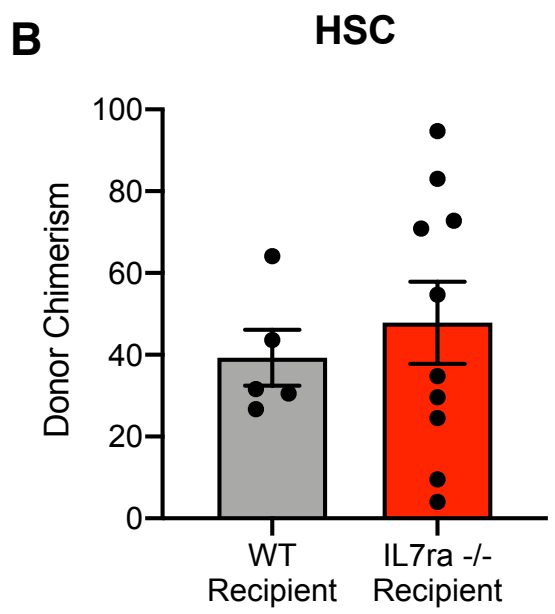
**B)** 500 WT, Flk2$^{-/-}$, IL7Rα$^{-/-}$ or FIDKO HSCs were transplanted into sublethally irradiated fluorescent WT recipients (mTmG or UBC-GFP) and donor chimerism of peripheral blood CD3 + T cells was quantified 16 weeks post transplantation. Reconstitution of T cells was significantly impaired upon transplantation of IL7Rα$^{-/-}$ (red bars) and FIDKO HSCs (red/white bars) compared to WT HSCs (grey bars). Data from the same mice as in Figure 3.

**C-C')**. 500 fluorescent WT HSCs (UBC-GFP), were transplanted into sublethally irradiated non-fluorescent WT or IL7Rα$^{-/-}$ recipients. Donor contribution to peripheral blood CD3+ T cells was quantified 16 weeks post-transplantation. **C)** Donor-derived and **C')** total T cells were not changed in an IL7Rα$^{-/-}$ recipient compared to a WT recipient. Data from the same mice as Figure 4.

**D)** Cellularity of peripheral blood CD3+ T cells in WT or IL7Rα$^{-/-}$ mice (solid bars) are compared to WT and IL7Rα$^{-/-}$ mice reconstituted with WT HSCs (pattern bars).

Differences in A-C') were analyzed with two-tailed Student t-test relative to WT mice, and differences in D) were analyzed across all four conditions by one-way ANOVA (p=0.0037) and Dunnett multi-parameter test. *, P<0.05; **, P< 0.005; ***, P< 0.0005; ****, P< 0.00005.



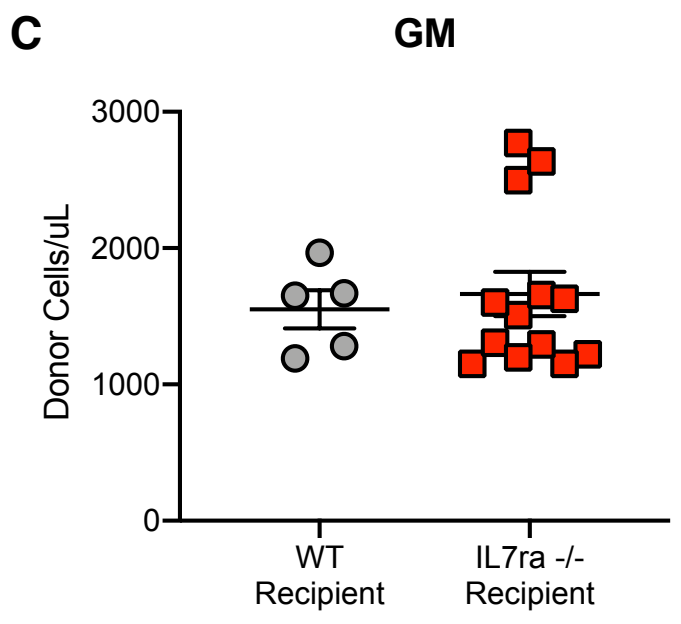



**Fig. S3. HSC engraftment was robust, regardless of donor HSC or recipient genotype.**
**A)** 500 WT, Flk2$^{-/-}$, IL7Rα$^{-/-}$ or FIDKO HSCs were transplanted into sublethally irradiated fluorescent WT recipients (mTmG or UBC-GFP) and donor chimerism of peripheral blood Granulocyte/Macrophages (GMs) cells was quantified 16 weeks post transplantation. Reconstitution of GMs was not significantly impaired upon transplantation of Flk2$^{-/-}$(white bars), IL7Rα$^{-/-}$ (red bars), or FIDKO HSCs (red/white bars) compared to WT HSCs (grey bars). Data from the same mice as in Figure 3.
**B,C).** 500 fluorescent WT HSCs (UBC-GFP), were transplanted into sublethally irradiated non-fluorescent WT or IL7Rα-/- recipients. Donor contribution to peripheral blood GMs and bone marrow HSCs was quantified 16 weeks post-transplantation. Data from the same mice as in Figure 4. **B)** Donor chimerism of bone marrow HSCs was similar in IL7Rα$^{-/-}$ compared to WT recipients. **C)** Donor-derived GM cells/uL of blood was similar in IL7Rα$^{-/-}$ compared to WT recipients. Statistics by two-tailed Student t-test. **, P< 0.005.

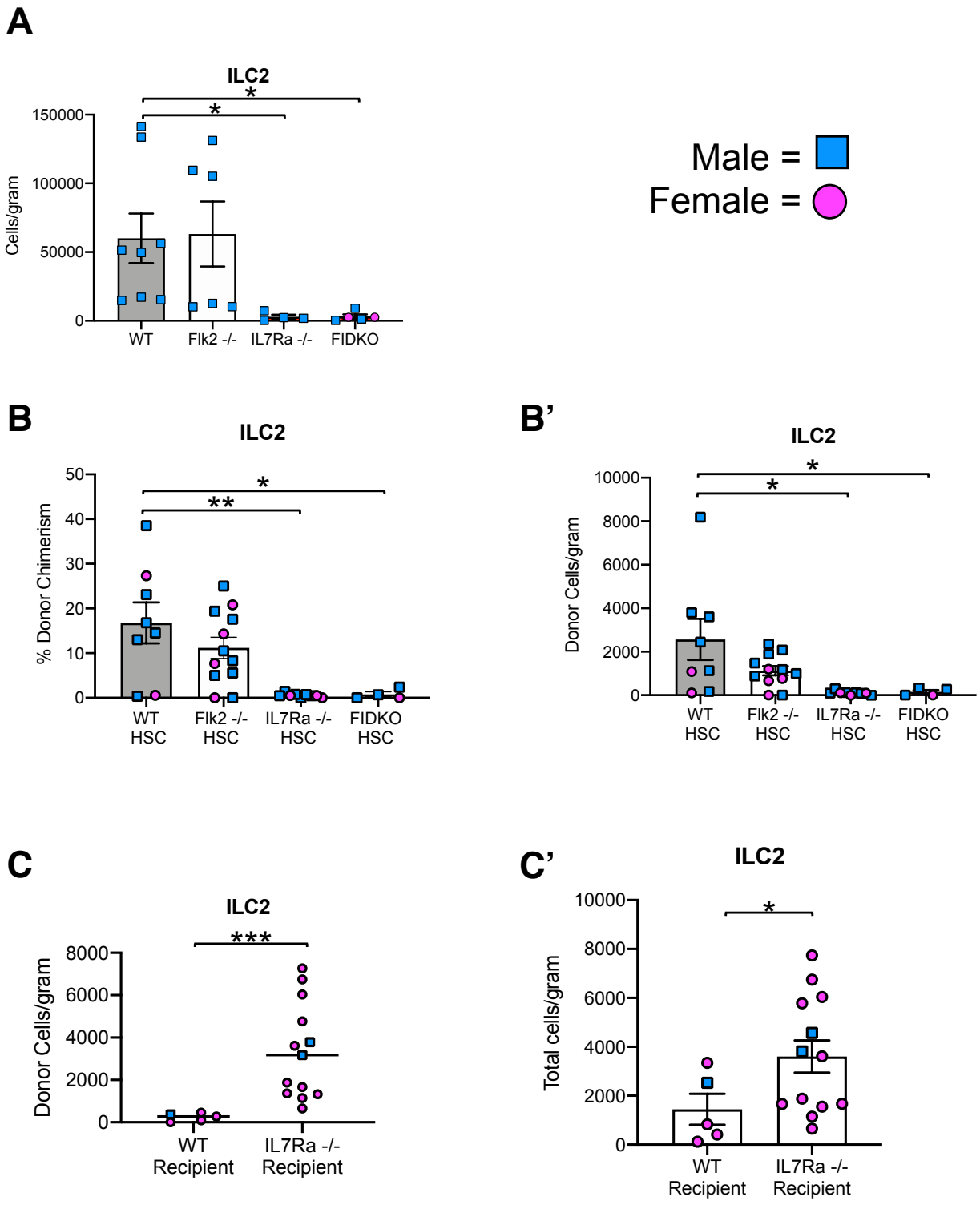



**Fig. S4. ILC2s in the lung were similarly affected in male and female mice.**

Data from figures 2, 3 and 4, with sex designation of each datapoint. Blue squares denote male mice and pink circles denote female mice.

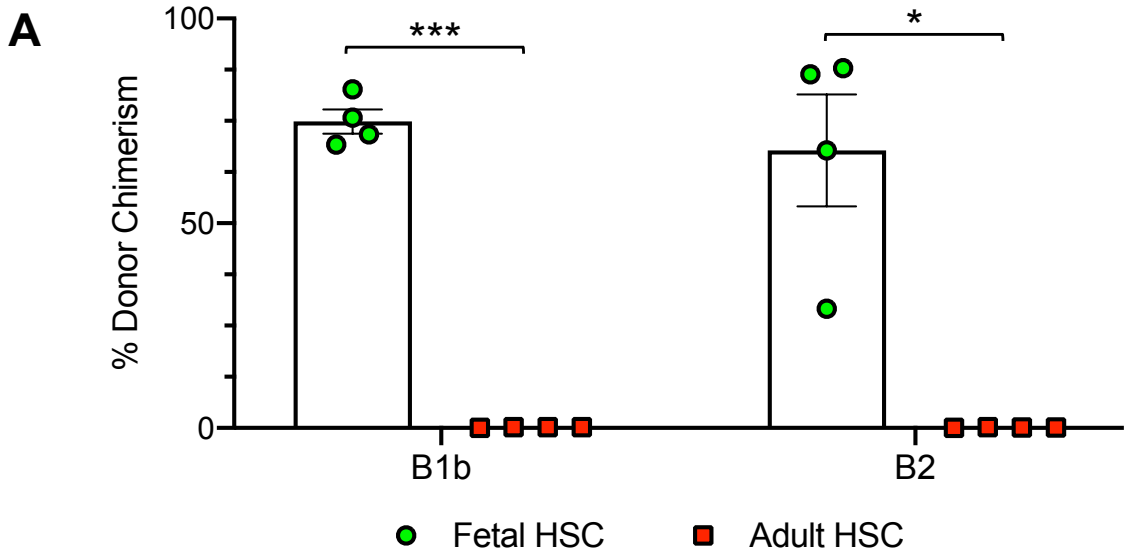
**A)** Quantification of ILC2s in WT, Flk2$^{-/-}$, IL7Rα$^{-/-}$, and FIDKO mice. Data from Figure 2.

**B)** Donor Chimerism and

**B')** quantification of donor ILC2s in from WT HSC, Flk2$^{-/-}$ HSC, IL7Rα$^{-/-}$ HSC, and FIDKO HSC mice. Data from Figure 3.

**C)** Quantification of donor-derived ILC2s and

**C')** quantification of total (host+donor) ILC2 numbers in WT and IL7Rα$^{-/-}$ recipients. Data from Figure 4.



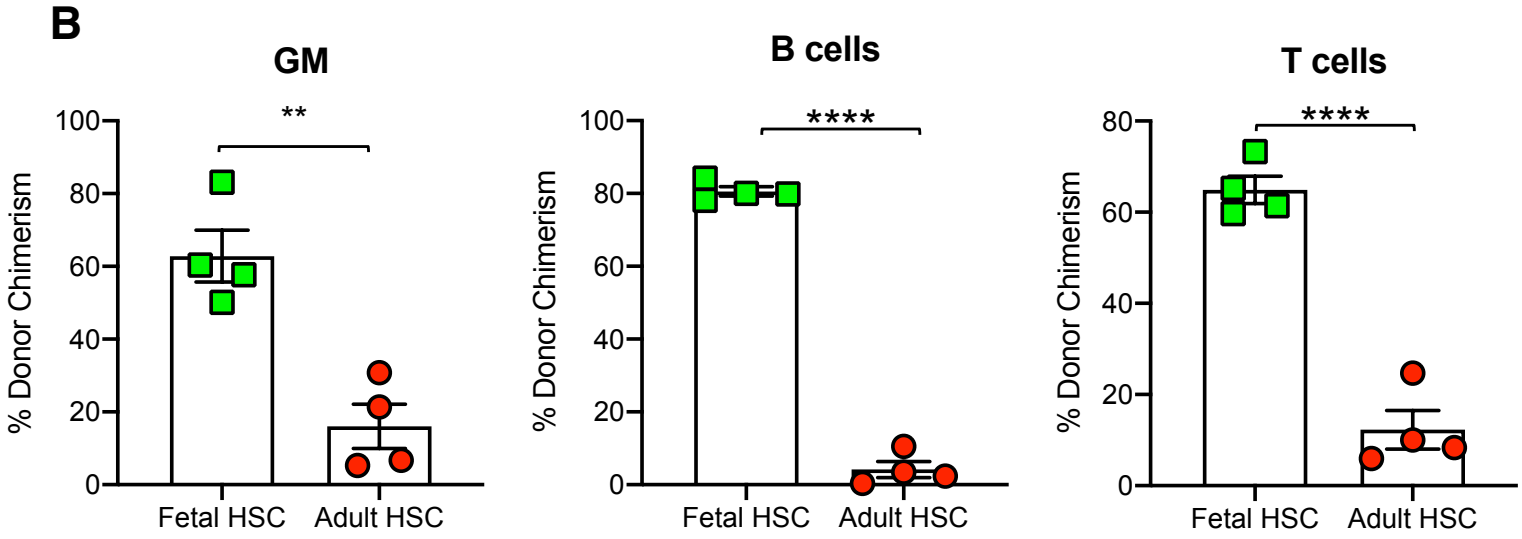




**Fig. S5. Fetal HSCs had greater mature cell reconstitution capacity compared to adult HSCs.** 250 fetal HSCs and 250 adult HSCs from different fluorescent mice (KuO and UBC- GFP, respectively) were co-transplanted into sublethally irradiated WT recipients and donor contribution to mature cells was quantified >16 weeks post transplantation.

**A)** Donor chimerism of peritoneal B1b and B2 was significantly greater by fetal HSCs compared to adult HSCs.

**B)** Donor chimerism of peripheral blood GMs, B cells and T cells was significantly greater by fetal HSCs compared to adult HSCs. Data from the same mice as in Figure 6. Differences were analyzed with two-tailed Student t test *, P<0.05; **, P< 0.005; ***, P<0.0005.

## Table S1. Antibodies used in experiments

| Antibody | Source | Identifier | Dilution |
|---|---|---|---|
| B220 - APC Cy7 | Biolegend | 103224 | 1:400 |
| CD3- APC | Biolegend | 100236 | 1:200 |
| CD4 - BV605 | Biolegend | 100451 | 1:400 |
| CD5 - APC | Biolegend | 100625 | 1:200 |
| CD11b - PB | Biolegend | 101223 | 1:400 |
| CD11b- PECy7 | Biolegend | 101216 | 1:400 |
| CD21 - PE Cy7 | Ebiosciences | 25-0211-82 | 1:200 |
| CD23 - PB | Biolegend | 101616 | 1:200 |
| CD25 - APC Cy7 | Biolegend | 102026 | 1:200 |
| CD45.2 - PB | Biolegend | 109820 | 1:400 |
| CD61-Alexa 647 | Biolegend | 104314 | 1:400 |
| CD150 - BV786 | Biolegend | 115937 | 1:400 |
| cKit - APC Cy7 | Biolegend | 105826 | 1:800 |
| Flk2 - APC | Biolegend | 135310 | 1:100 |
| FoxP3 - PB | Biolegend | 126409 | 1:100 |
| Gr1-Pacific Blue | Biolegend | 108430 | 1:400 |
| IgM - BV605 | Biolegend | 406523 | 1:400 |
| KLRG - BV605 | Biolegend | 138419 | 1:100 |
| Sca1 - PB | Biolegend | 122520 | 1:400 |
| TCRB - Pe Cy7 | Biolegend | 109222 | 1:200 |
| Ter119-PECy5 | Biolegend | 116210 | 1:800 |

| Antibody | Source | Identifier | Dilution |
|---|---|---|---|
| CD3 - A700 | Biolegend | 100216 | 1:100 |
| CD4 - A700 | Biolegend | 100429 | 1:400 |
| CD5 - A700 | R&D Systems | FAB115N-025 | 1:400 |
| CD8 - A700 | Biolegend | 300919 | 1:200 |
| Ter119 - A700 | Biolegend | 116220 | 1:400 |
| B220 - A700 | Biolegend | 103231 | 1:400 |
| Gr1 - A700 | Biolegend | 108421 | 1:400 |
| CD11b - A700 | Biolegend | 101222 | 1:800 |
| CD3 - biotin | Biolegend | 100243 | 1:200 |
| CD4 - biotin | Biolegend | 100403 | 1:200 |
| CD5 - biotin | Biolegend | 100603 | 1:400 |
| CD8 - biotin | Biolegend | 100703 | 1:200 |
| Ter119 - biotin | Biolegend | 116203 | 1:400 |
| Nk1.1 - biotin | Biolegend | 108703 | 1:200 |
| CD19 - biotin | Biolegend | 115503 | 1:400 |
| F4/80 - biotin | Biolegend | 123105 | 1:400 |
| FcεRIα - biotin | Biolegend | 101303 | 1:200 |
| STA - BV786 | Biolegend | 405249 | 1:400 |