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Technical Comment

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Fast and efficient genetic engineering of hematopoietic precursor cells for the study of dendritic cell migration

Dendritic cells (DCs) are sentinels of the adaptive immune system that reside in peripheral organs of mammals. Upon pathogen encounter, they undergo maturation and up-regulate the chemokine receptor CCR7 that guides them along gradients of its chemokine ligands CCL19 and 21 to the next draining lymph node. There, DCs present peripherally acquired antigen to naïve T cells, thereby triggering adaptive immunity [1, 2]. Their high migratory speed and chemotactic prowess, the relative ease to generate large cell numbers in culture and their suitability for in vitro and in vivo assays has made DCs one of the most powerful tools to study cell migration [3]. Typically, DCs are differentiated from un-fractionated bone marrow (BM) in the presence of differentiation-promoting factors such as granulocyte-macrophage colony stimulating factor (GM-CSF) [4]. Once mature, these terminally differentiated BM DCs have a very limited lifespan, impeding stable genetic modifications. Consequently, research is hampered by time- and resource-consuming generation of genetic mouse models.

To overcome these limitations, several methods have been developed to obtain long-term DC cultures. This has been either achieved by differentiation of DCs from embryoid bodies [5], oncoprotein driven immortalization of DCs [6–9], or growth factor dependent, long-term culture of splenic DCs [10]. Although migratory properties are considered to be one of the hallmarks of DC biology, we never observed efficient migration in any of these cultures (personal observation).

Recently, Flt3L expanded hematopoietic precursors, transiently immortalized via a retrovirally delivered, estrogen inducible form of the transcription factor Hoxb8 have been introduced (Hoxb8-FL cells) [11]. Upon estrogen withdrawal and GM-CSF culture the resulting DCs closely resemble BM DCs in their transcriptome, cytokine secretion and priming of naïve T cells (Fig. 1A) [11].

For direct comparison we differentiated DCs from BM and Hoxb8-FL cells side-by-side in the presence of GM-CSF [4]. Cells from both origins expressed high levels of the DC markers Cd11c and major histocompatibility complex II (MHCII, Fig. 1B). Importantly, Hoxb8-FL DCs expressed CCR7 [2], albeit at slightly lower levels as compared to BM DCs (Fig. 1B). When incorporated into 3D collagen gels [12] and recorded by time-lapse video microscopy both fractions showed vivid protrusion dynamics and migrated persistently for several micrometers before rounding up and changing direction (Supporting Information Movie 1). Migratory speeds of Hoxb8-FL DCs were slightly reduced as compared to BM DCs (Fig. 1C). When exposed to gradients of CCL19, HoxB8-FL DCs showed a strong chemotactic response with a slightly reduced chemotactic index compared to BM DCs, potentially attributable to lower levels of CCR7 (Fig. 1D and E and Supporting Information Movie 1). Finally, we co-injected differently labeled DCs of both origins into mouse footpads and found that they arrive in comparable numbers in the popliteal lymph node 24 h after injection (Fig. 1F). Taken together, Hoxb8-FL DCs showed robust migratory behavior and chemotaxis in vitro and in vivo.

Next, we set out to probe the tractability for genetic modifications via the CRISPR/Cas9 system. As a proof of principle, we first assessed if a fluorescent reporter (GFP) could be deleted in undifferentiated Hoxb8-FL cells. Hoxb8-FL cells, homozygous for membrane GFP (mG), were infected with lentiviruses coding for puromycin resistance, Cas9 and a guide RNA (gRNA) [13] directed against GFP (anti-GFP-1 or 2) or a scrambled (scrbl) control. GFP negative cells could be detected 3 to 4 days after infection while negligible signal was detected in the GFP negative gate of scrbl infected cells (Fig. 1G). To determine the efficiency of the CRISPR/Cas9 system in Hoxb8-FL cells, we exposed infected mG cells to antibiotic selection and expanded survivors. We found that more than 90% of mG cells infected with gRNA anti- GFP-1 were GFP negative. For gRNA anti-GFP-2 this effect was even stronger, with almost 100% of cells in the GFP negative gate. In contrast, mG cells infected with the scrbl gRNA lentivirus retained their fluorescence (Fig. 1H). These results demonstrate that CRISPR/Cas9 mediated genome editing in HoxB8-FL progenitors is highly efficient.

Next, we wanted to explore if activity of the CRISPR/Cas9 system interferes with the ability of Hoxb8-FL cells to differentiate to DCs and if endogenous genes can be targeted with comparable efficiency. Initially, we focused on the gene *Itgb2*, coding for the leukocyte specific integrin beta 2. Together with integrin alpha x

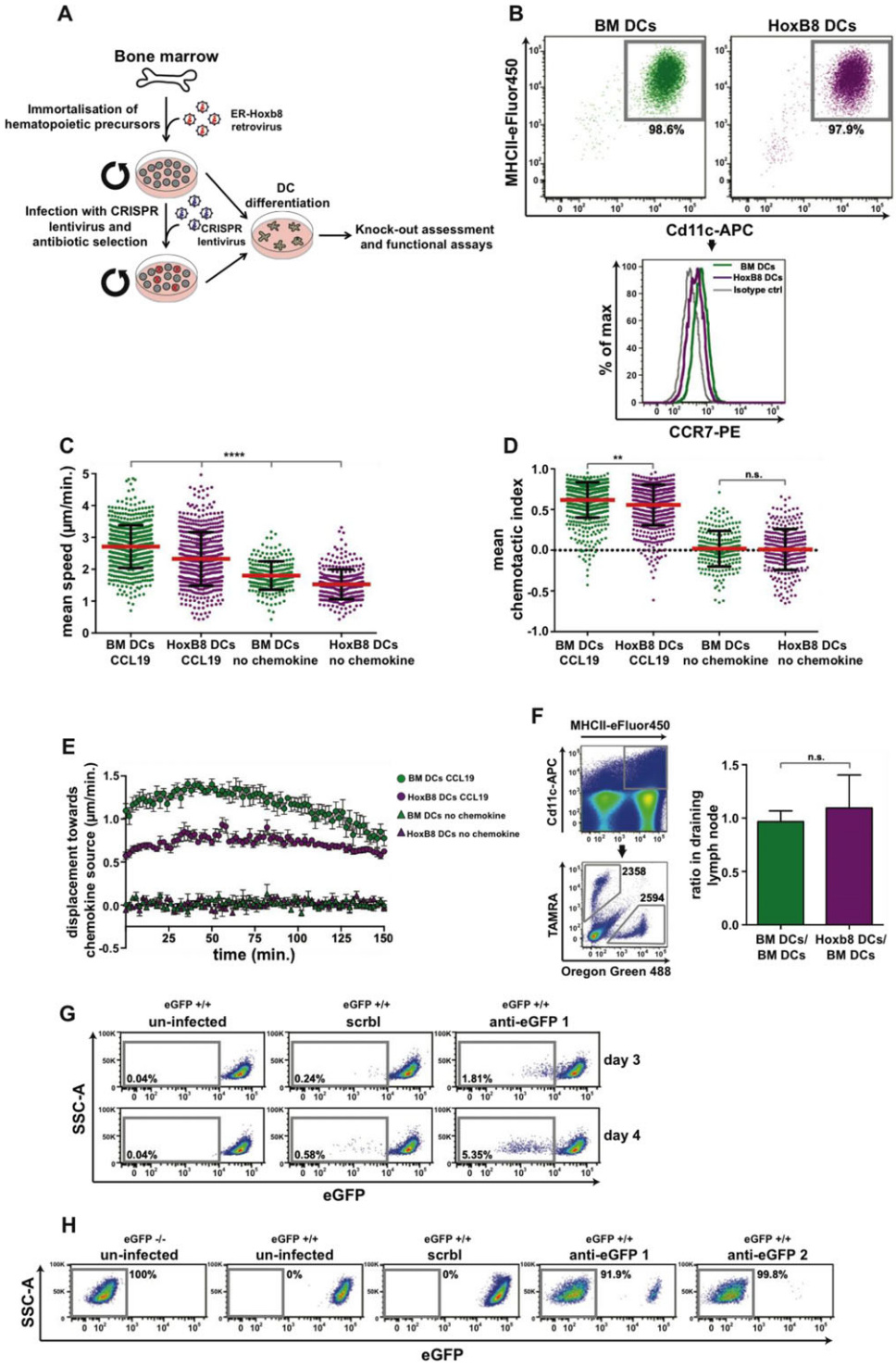

Figure 1. Migratory properties of BM- and Hoxb8-FL DCs in vitro and in vivo. (A) Schematic of generation, maintenance, LentiCRISPR infection and differentiation to DCs of Hoxb8-FL cells (B) FACS staining of DCs for Cd11c, MHCII and CCR7. CCR7 levels are pre-gated on Cd11c/MHCII high population (gray boxes). (C) Mean single cell speeds (µm/min.) of DCs migrating in 3D collagen gels in presence or absence of CCL19 gradient. Mean (red line) ± SD (black bars). Kruskal–Wallis test, $^{****}p < 0.0001$, $n = 663/567$ (BM-/Hoxb8 DCs CCL19), $n = 235/299$ (BM-/Hoxb8 DCs no chemokine), pooled from three biologically independent experiments. (D) Mean single cell chemotactic indices of cells in (C). Kruskal–Wallis test, $^{**}p < 0.01$. (E) Average y-displacement (µm/min.) over time (min.) of BM- and Hoxb8-FL DCs in the presence (circles) or absence (triangles) of CCL19 gradient. Mean (dots/triangles) ± SEM (black bars). $n = 6$ collagen gels (CCL19) or $n = 3$ collagen gels (no chemokine), pooled from three biologically independent experiments. (F) *left*: gating strategy to calculate the ratio of DCs in the draining lymph node 24 h after injection shown on the *right*. SD (black bars). unpaired *t*-test, not significant, $p = 0.3415$. $n = 6$ (BM/BM) or $n = 17$ (Hoxb8/BM), pooled from two biologically independent experiments. (G) eGFP expression levels of mG Hoxb8-FL cells 3 or 4 days after infection with indicated lentiviruses. (H) eGFP expression levels of mG or ctrl Hoxb8-FL cells after infection with indicated lentiviruses and antibiotic selection.

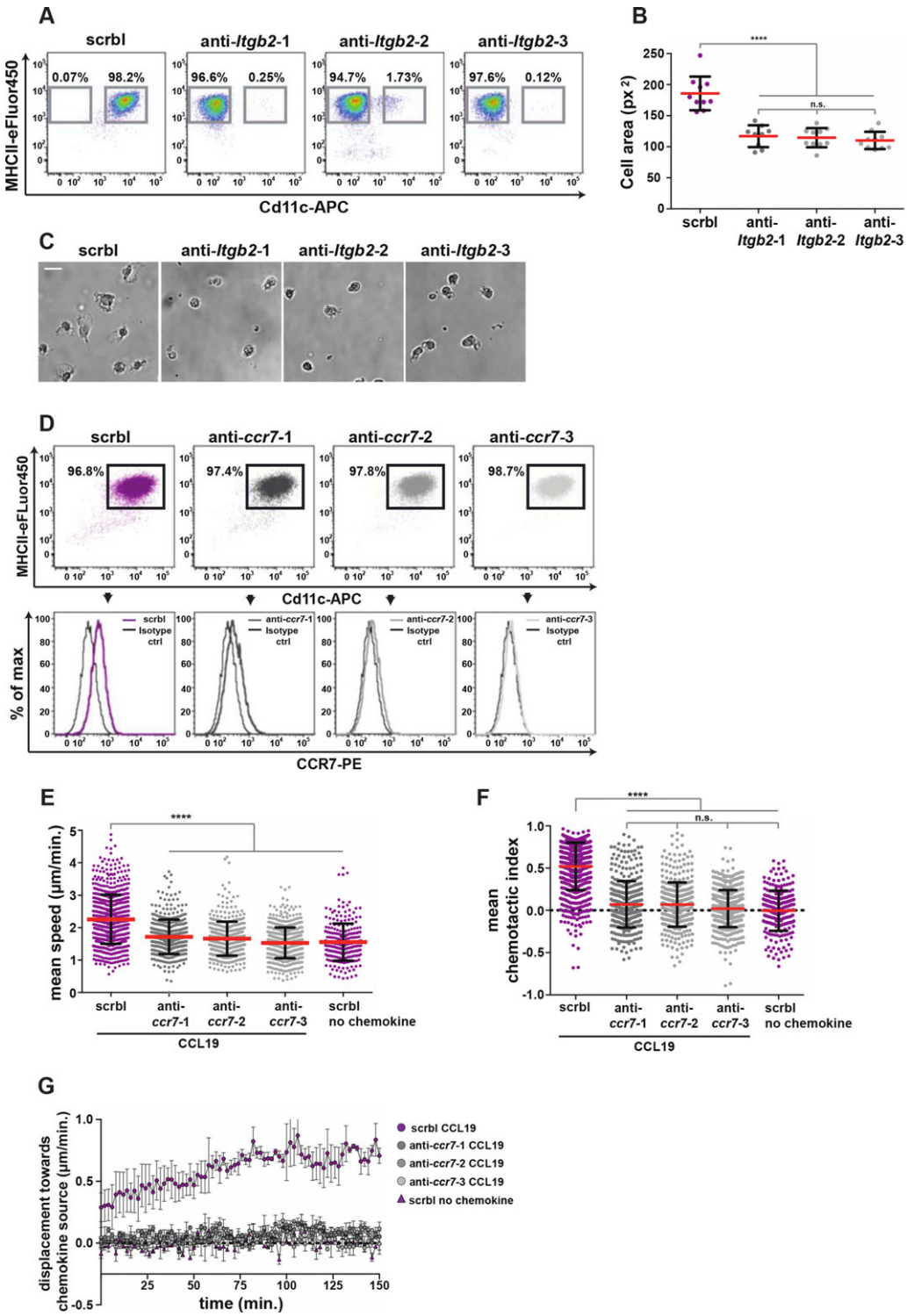

Figure 2. Characterization of Hoxb8-FL *Itgb2* and *ccr7* knockout DCs. (A) FACS staining of putative *Itgb2* knockout DCs for Cd11c and MHCII. (B) Cell area (pixel^2) of putative *Itgb2* knockout DCs, plated on CCL21. Mean (red line) ± SD (black bars). ANOVA, $^{****}p < 0.0001$. $n = 10$ cells from each batch. (C) Snapshots of putative *Itgb2* knockout DCs plated on CCL21. (D) FACS staining of putative *ccr7* knockout DCs for Cd11c, MHCII and CCR7. CCR7 levels are pre-gated on Cd11c/MHCII high population (black boxes). (E) Mean single cell speeds (µm/min.) of putative *ccr7* knockout DCs migrating in 3D collagen gels in CCL19 gradient. Mean (red line) ± SD (black bars). Kruskal–Wallis test, $^{****}p < 0.0001$, $n = 755/421/406/574/256$ (scrbl/anti-CCR7- 1/2/3/scrbl no chemokine), pooled from three biologically independent experiments. (F) Mean single cell chemotactic indices of cells in (E). Kruskal–Wallis test, $^{****}p < 0.0001$. (G) Average y-displacement (µm/min.) over time (min.) of putative *ccr7* knockout DCs in the presence (circles) or absence (triangles) of CCL19 gradient. Mean (dots/triangles) ± SEM (black bars). $n = 3$ collagen gels (CCL19) or $n = 2$ collagen gels (scrbl, no chemokine), pooled from three biologically independent experiments.

it forms the trans-membrane heterodimer DC marker Cd11c. Beta 2 integrins mediate cell-substrate adhesions and couple the contractile force of the cytoskeleton to the environment [14]. We designed three different gRNAs (anti-*Itgb2*- 1–3) directed against *Itgb2*, produced lentiviruses and infected HoxB8-FL cells. After antibiotic selection, cells were expanded and differentiated to DCs. Putative *Itgb2* knockout and control cells were morphologically indistinguishable in suspension (data not shown) and cells infected with the scrbl gRNA showed high Cd11c and MHCII levels (Fig. 2A). DCs derived from cells infected with anti-*Itgb2* showed no Cd11c staining, while retaining high MHCII levels (Fig. 2A). To obtain a functional read-out we coated glass-slides with CCL21, which triggers integrin beta 2 dependent adhesion, polarization and migration trough CCR7 [15]. DCs derived from anti-*Itgb2* Hoxb8-FL cells failed to adhere to CCL21 coated glass slides (Fig. 2B and C and Supporting Information Movie 2).

As a second endogenous candidate we chose chemokine receptor CCR7. We designed three, *ccr7* specific- (anti-*ccr7*) and one scrambled control gRNA. As in the above-described experiments we observed a relatively low number of infected Hoxb8-FL cells surviving puromycin selection, we compared blasticidin as an antibiotic marker and found that survival rates are increased (data not shown). DCs from all infections showed normal Cd11c and MHCII levels (Fig. 2D). Importantly, cells from all anti-*ccr7* infections showed a drop in their CCR7 levels. In case of anti-*ccr7*-2 and 3, it was almost reduced to isotype control levels, suggesting near complete knockouts (Fig. 2D). In 3D collagen gels DCs derived from anti-*ccr7* infections showed a significantly lower mean speed and entirely failed to migrate toward a CCL19 source (Fig. 2E–G and Supporting Information Movie 3) as would be expected for CCR7 knockouts that are unresponsive to CCL19.

Taken together, we demonstrate that knockouts can be easily and efficiently generated in Hoxb8-FL cells. These cells can be further differentiated to DCs that behave very similar to their BM derived counterparts. Recently, it has been observed that BM GM-CSF cultures, under certain culture conditions, comprise a heterogeneous population of macrophages and DCs, both expressing Cd11c and different levels of MHCII [16]. In our hands GM-CSF cultures from both BM and Hoxb8 origin, express homogenous levels of MHCII and CCR7 (which is not expressed by macrophages [16]) and thus show a homogenous and complete chemotactic response pattern. Genome editing efficiency was high in our hands, although for some gRNAs a certain small percentage of cells retained detectable protein. Residual expression can be easily circumvented by, as shown here, designing multiple gRNAs or by single cell cloning that will also facilitate thorough characterization of the genetic changes. The high efficiency of CRISPR/Cas9 mediated gene knockouts might also allow for infections of Hoxb8-FL cells with pooled gRNA libraries for specific subsets of genes, followed by functional screens. To gain complete control over the Hoxb8-FL genome we are currently establishing knock-in strategies to tag, replace and mutate endogenous genes. This is ideally done with non-integrating vectors, which is challenging due to the relative resistance of Hoxb8-FL cells to transient transfection. Notably, the potential of Hoxb8-FL cells to differentiate into other cell types of the myeloid and lymphoid lineage (e.g. Flt3 DCs, macrophages, B cells and T cells [11]) allows broader utilization of the targeted precursor cells.

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Abbreviations: **BM**: bone marrow · **Cas9**: CRISPR associated protein · **CRISPR**: clustered regularly interspaced short palindromic repeats · **DCs**: dendritic cells · **GM-CSF**: granulocyte-macrophage colony stimulating factor · **gRNA**: guide RNA

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