Classical Flt3L-dependent dendritic cells control immunity to protein vaccine

Niroshana Anandasabapathy,1,2,6 Rachel Feder,1,2 Shamim Mollah,3 Sze-Wah Tse,4 Maria Paula Longhi,1,2 Saurabh Mehandru,1,2 Ines Matos,1,2 Cheolho Cheong,1,2 Darren Ruane,1,2 Lucas Brane,1,2 Angela Teixeira,1,2 Joseph Dobrin,1,2 Olga Mizenina,1,2 Chae Gyu Park,1,2 Matthew Meredith,2,4 Björn E. Clausen,5 Michel C. Nussenzweig,2,4 and Ralph M. Steinman1,2

DCs are critical for initiating immunity. The current paradigm in vaccine biology is that DCs migrating from peripheral tissue and classical lymphoid-resident DCs (cDCs) cooperate in the draining LNs to initiate priming and proliferation of T cells. Here, we observe subcutaneous immunity is Fms-like tyrosine kinase 3 ligand (Flt3L) dependent. Flt3L is rapidly secreted after immunization; Flt3 deletion reduces T cell responses by 50%. Flt3L enhances global T cell and humoral immunity as well as both the numbers and antigen capture capacity of migratory DCs (migDCs) and LN-resident cDCs. Surprisingly, however, we find immunity is controlled by cDCs and actively tempered in vivo by migDCs. Deletion of Langerin+ DC or blockade of DC migration improves immunity. Consistent with an immune-regulatory role, transcriptomic analyses reveals different skin migDC subsets in both mouse and human cluster together, and share immune-suppressing gene expression and regulatory pathways. These data reveal that protective immunity to protein vaccines is controlled by Flt3L-dependent, LN-resident cDCs.

Flt3L is a DC hematopoietin that maintains DC numbers at set levels throughout adult life (Liu et al., 2007, 2009) and at sites relevant to vaccination, including the skin and skin-draining LN (Brasel et al., 1996; Maraskovsky et al., 1996). In healthy individuals, Flt3L is tightly regulated and at the limits of detection by ELISA; notably, it is 20-fold lower than CSF-1 or c-kit ligand (Shadle et al., 1989; Langley et al., 1993; Lyman and McKenna, 2003). Flt3L is secreted during acute infection, however, leading to DC-mediated support of NK function (Eidenschenk et al., 2010; Guermonprez, 2012). During s.c. immunization, the composition of DC subsets in the skin-draining LNs is transiently altered (Kastenmüller et al., 2011). It is unknown if Flt3L...
is secreted during immunization to regulate DC expansion acutely or if Flt3 signaling is required for productive immunity.

Flt3L and its receptor (Flt3, FLK2) instruct progenitors along a DC developmental pathway regulating the mobilization of preDCs from the blood to give rise to IFN-α-producing PDC, CD8α−, and CD8α+εcDCs in lymphoid organs and tissue-resident DCs such as Langerin+CD103+ DCs in skin (Waskow et al., 2008). Lymphoid CD8α+ (Bozzacco et al., 2010) and tissue CD103+ DCs both cross-present antigens (the major pathway of tumor and viral antigen presentation), derive from preDCs (Ginhoux et al., 2009), and share Flt3L developmental dependence (Liu et al., 2009), with common regulation downstream of Flt3 by mTOR (Sathaliyawala et al., 2010). These findings suggest DC ontogeny may dictate function, one rationale for the use of hematopoietics to selectively drive DC development for clinical use. Flt3L is being reintroduced to the clinic to potentiate human vaccines. It is unclear if bias by Flt3L to cross-presenting DCs from skin and LN may be exploited for protein-based vaccine delivery. Also, Langerin+CD103+ DCs, which are tissue-resident migDCs originating from skin, are specialized to cross-present viral antigens to T cells during cytolytic infection (Bedoui et al., 2009a). However, their role in immunization to viral antigens has not been established.

We observe Flt3 is required for robust immunity to s.c. immunization and can enhance immunity. Surprisingly, we find that irrespective of Flt3L treatment, migDCs in the LN (including Langerin+CD103+ cross-presenting DCs) are not required for CD4+ T cell effector function, despite having greater efficiency of s.c. protein capture in the LN than resident CD8α cDCs. Impairing DC migration from skin to the sDLN via knockout of the CCR7 receptor and deletion of migDC subsets including Langerin+CD103+ DCs enhanced, not diminished, immune priming. Rather, the immune response develops through CD11c+ZBTB46-dependent cDCs. Transcriptomics in mouse and human reveal migDC subsets from skin relate most closely to each other and share gene signatures related to dampening of DC and T cell activation. Thus, we demonstrate that the immune response is controlled by cDCs in lymphoid tissue and that tissue microenvironment may confer immunosuppressive DC function in vivo.

RESULTS

s.c. immunity is Flt3L dependent

To assess if Flt3L is detected after immunization, we compared serum murine Flt3L levels by ELISA before and after immunization with adjuvant and a vaccine that targets protein antigens to DCs in vivo using antibodies directed against the C-type lectin CD205. Elevated Flt3L was detected in serum within 1 h of administering adjuvant with 0.5 µg of HIV gag p24-αCD205 fusion antibody (αCD205-gag p24; Fig. 1 A). As adjuvant, we tested both GLA-SE (glucopyranosyl lipid adjuvant-stable emulsion), which acts through TLR4 (Duthie et al., 2011; Pantel et al., 2012), and the adjuvant polyIC(LC) (polynosinic-polycytidylic acid stabilized with polylysine double-stranded RNA and carboxymethylcellulose), which is a double-stranded DNA mimic that signals through TLR3 (Schulz et al., 2005) and MDA5 (Gitlin et al., 2006). To assess if Flt3 signaling was required, we compared prime-boost immunization with 0.5 µg αCD205–gag p24 vaccine with GLA-SE in control versus Flt3−/− mice. Flt3−/− mice consistently had 50% lower vaccine responses (Fig. 1 B). Collectively, these data suggest Flt3L is secreted after adjuvant administration with protein immunization and Flt3 signaling is required for optimal immunity.

To assess the impact of Flt3L on s.c. immunization, we treated mice with Flt3L or PBS surrounding prime-boost immunization with GLA-SE (Fig. 1 C). Upon antigen recall, we observed markedly increased IFN-γ production in CD4+ T cell isolated from spleen when challenged with HIV-gag p24, but not control p17 peptides (Fig. 1 D and Fig. S1, gating). Flt3L–treated mice had enhanced antigen–specific clonal expansion in vitro (CFSElow, IFN-γ+) and enhanced CD4+ T cell IFN-γ responses ex vivo when isolated from draining LNs, lung parenchyma, and small intestinal lamina propria as assayed by intracellular cytokine staining (ICS). Improved titers of serum IgG directed against gag p24 were observed. To determine if Flt3L-improved protein immunization is adjuvant specific, we also tested polyIC(LC) as adjuvant (Fig. 1 E). We again observed enhanced CD4 IFN-γ recall, CD4+ clonal expansion, mucosal immunity, and HIV gag p24-specific IgG titers.

To determine if Flt3L potentiates both soluble and DC-targeted protein vaccines, we immunized mice with soluble versus αCD205–targeted gag p24 and GLA-SE. Flt3L improved soluble and DC-targeted responses (Fig. 2 A). At high antigen dose (5 µg) irrespective of Flt3L treatment, no difference was observed when comparing soluble to targeted vaccine responses. However, at 0.5 µg, antibody–mediated antigen targeting imparted a significant benefit to immunization. This effect was not dose-sparing, as targeted vaccination irrespective of Flt3L treatment resulted in improved clonal proliferation with respect to soluble antigen (Fig. 2 B). Similar trends were noted in mucosa and LNs (unpublished data). Thus, Flt3L improves both DC-targeted and nontargeted protein immunization. We established that targeting specificity through αCD205 occurred at 0.5 µg as soluble protein immunization lacked a discernible response at 0.5 µg (Fig. 2 A and B).

Although αCD205–gag p24 priming in primates induces both CD4 and CD8 immunity, in B6 mice we observe only CD4+ priming due to epitope restriction (Trumpfheffer et al., 2008). To assess Flt3L treatment on cross-presentation to CD8+ T cells, we examined polyclonal CD8+ T cell responses after immunization to αCD205–OVA with polyIC(LC). CD8+ T cell IFN-γ was assayed using mixed OTI and II recall peptides and p24-pooled peptide controls. When assayed from lung parenchyma, spleen, and lamina propria, T cells from Flt3L mice had increased CD8+ T cell IFN-γ production, proliferation, and IgG titers to OVA (Fig. 2 C). Therefore, we conclude Flt3L enhanced cross-presentation to CD8+ T cells.

Flt3L expands CD205+ LN-resident cDCs and migratory DCs and enhances in vivo αCD205–based antigen capture

To better understand the possible mechanisms underlying Flt3L enhancement of s.c. immunization, we examined the effect of...
and intracellular CD205 staining). In vitro CD8α cDC capture of CD205 was almost 100% efficient at 1 and 3 h, and the percentage of cDCs that captured CD205 in vitro was increased by Flt3L due to higher representation of this population (Fig. 3 C; from 44 to 67%). This corresponded closely to the shift in total percentage of CD205+ cells (Fig. 3 B). Flt3L also contributed to an overall expansion of total migDCs.

Five phenotypically distinct migDC populations traffic from skin to draining LN (Henri et al., 2010a,b). Langerin+ Flt3L on DC subsets in the skin-draining LNs of Flt3L-treated, control, and Flt3L−/− mice (Fig. S2, A and B, gating). Flt3L markedly increased PDCs and cDCs in skin-draining LNs (Fig. 3, A and B). PDCs are CD205− and PDCA1+ (unpublished data). Within cDCs, we observed consistent expansion of CD8α+ CD11b+CD8α-CD11b+ cDC numbers. CD205+ CD8α+ cDCs comprise ~40% of CD8α+ cDCs in the skin-draining LNs. Upon Flt3L treatment, CD205+ CD8α+ cDCs cells were enriched to 73% of cDCs (Fig. 3 B; cell surface and intracellular CD205 staining). In vitro CD8α cDC capture of CD205 was almost 100% efficient at 1 and 3 h, and the percentage of cDCs that captured αCD205 in vitro was increased by Flt3L due to higher representation of this population (Fig. 3 C; from 44 to 67%). This corresponded closely to the shift in total percentage of CD205+ cells (Fig. 3 B). Flt3L also contributed to an overall expansion of total migDCs.

Five phenotypically distinct migDC populations traffic from skin to draining LN (Henri et al., 2010a,b). Langerin+...
After Flt3L treatment, a small decrease in in vitro capture of αCD205 by migDCs was apparent (from 75 to 69%; Fig. 3 E). To determine if migDCs and LN-resident cDCs in the nodes have equal access to antigen in vivo, we compared fluorescently labeled αCD205 capture 3 h after s.c. injection into Flt3L-treated and untreated mice (Fig. 3 F). MigDCs captured antigen with greater efficiency than cDCs by αCD205-A647 label in the proximal LNs (popliteal), irrespective of Flt3L treatment. Flt3L improved αCD205-A647 label by both migDCs in the distal LNs (inguinal) and cDCs in the proximal subsets include Langerhans cells (LCs), CD103+ Langerin+ dermal DCs, and the very rare CD103− Langerin+ DCs. Langerin− subsets include CD11b+ DCs and CD11b− DCs. Although some increase in Langerin+ CD103+ DCs occurred after Flt3L treatment, we observed the major expansion of migDCs was seen in the CD11b+ and CD11b− compartments (Fig. 3 D; Mollah et al., 2014). Thus, Flt3L biases cDCs in the cutaneous LN toward CD8αCD205+ differentiation and expands migratory CD11b+, CD11b−, and CD103+ Langerin+ DCs. MigDCs express high levels of CD205+ (Fig. 3 D).
Figure 3. Flt3L expands classical, migratory, and plasmacytoid DC subsets in the skin draining LN and enhances antigen capture by CD205+ migratory and LN-resident cDCs in vivo. (A) Flt3L-treated (left) compared with WT (middle) and Flt3L−/− mice (right). One representative experiment (of three) with sample quantitation of DC subsets for two pooled skin-draining LNs. Error bars show standard deviation between 3 mice. *, P ≤ 0.1; *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001. (B) Representative CD8α+ CD11b low and CD8α− CD11b high DC subset gating, from classical (cDCs) gate of skin draining LN from mice treated with Flt3L (left) or controls (right). Total % CD205 positive cells from within CD8α+ CD11b low cDC subset (cell surface and intracellular staining). (C) In vitro capture of αCD205 with equivalent total skin draining LN cells from Flt3L-treated versus PBS mice gated on total LN cDCs versus CD8α cDCs. One representative experiment of two with n = 3 mice per group, error bars depict SEM. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001. (D) Skin draining LN migratory DC subsets from within IAE+ CD11c+IAE− population from mice treated with Flt3L (left) or controls (right). CD103+ gating from within Langerin+ subset. CD205 cell surface and intracellular staining from migratory subsets (flow cytometry). (E) In vitro capture of αCD205+ with equivalent total skin draining LN cells from Flt3L-treated versus PBS mice, gated on migratory DCs. One representative experiment of 2 with n = 3 mice per group, error bars depict SEM. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001. (F) s.c. immunization does not depend on Langerin+ DCs, including Langerin+ CD103+ DCs. Previous work suggested that migratory DCs initiate immune priming in the LN (Itano et al., 2003; Allenspach et al., 2008), (popliteal) LNs (Fig. 3 F), suggesting improved antigen capture in vivo. Thus, in vivo migratory DCs in LNs captured and internalized αCD205 more rapidly than cDCs, and Flt3L improved antigen uptake by both.

s.c. immunization does not depend on Langerin+ DCs, including Langerin+ CD103+ DCs

Previous work suggested that migratory DCs initiate immune priming in the LN (Itano et al., 2003; Allenspach et al., 2008), (popliteal) LNs (Fig. 3 F), suggesting improved antigen capture in vivo. Thus, in vivo migratory DCs in LNs captured and internalized αCD205 more rapidly than cDCs, and Flt3L improved antigen uptake by both.
and we observed more rapid antigen capture by migDCs in LNs. Langerin 103+ DCs are dermal migDCs that cross-prime to virus (Bedoui et al., 2009b), yet a role in viral vaccine priming has not been established. To address this, we used Langerin-diphtheria toxin (DT) receptor (DTR) mice (LDTR), where expression of the DTR in Langerin+ cells permits depletion of Langerin+ DCs by DT treatment (Noordegraaf et al., 2010). In LDTR mice, a single dose of DT leads to deletion in the skin-draining LN of some Langerin+ CD8α+ cDCs (Kissenpfennig et al., 2005), and all CD103+ Langerin+ dermal DCs and LCs (Bennett et al., 2005; Noordegraaf et al., 2010). Robust depletion of Langerin+ migDCs occurred after DT administration in both untreated and Flt3L-treated mice including LC and CD103+ migDCs (Fig. 4 A and B). DT was administered 3 d and 1 d before vaccination during prime and boost phases (schema, Fig. 4 C). At 5 µg of αCD205-gag p24 with adjuvant, all Langerin+ DCs were dispensable for efficient vaccine priming at baseline, and their deletion did not diminish CD4+ vaccine responses, irrespective of Flt3L treatment when compared with controls administered DT at the same dosing scheme. In some Flt3L-treated groups and in all PBS controls (LNs, Spleen), we were surprised to note significantly higher direct ex vivo effector responses in LDTR mice versus control mice. To ensure s.c. immunization did not by-pass migratory DCs, we examined low-dose αCD205-gag p24 i.d. immunization into the flank (Fig. 4 C). Mice lacking CCR7 had significantly higher CD4+ T cell IFN-γ levels in the draining proximal LN (P < 0.0001) and distally in spleen (P < 0.05) and nondraining LNs (unpublished data). These data suggested that blockade of CCR7-dependent DC migration to the LN enhances, not impairs, immunity.

To determine whether migratory DCs were requisite for CD8 immunity, we also tested s.c. αCD205-OVA immunization. Again, no loss of CD8+ T cell proliferation in CCR7 KO mice was observed (Fig. 5 D). CCR7 KO mice may have defects in T reg cell activity that could support enhanced priming (Menning et al., 2007). To restrict CCR7 deficiency to DCs, we generated mixed bone marrow chimeras with CD45.1 and ZBTB46 DTR or CCR7 and ZBTB46DTR. Zbtb46 is a recently described cDC-specific transcriptional factor that distinguishes classical DCs from other mononuclear phagocytic lineages and from plasmacytoid DCs (Meredith et al., 2012; Satpathy et al., 2012). When uncompensated CCR7 loss was restricted to migDCs, there was no loss of immunity (Fig. 5 E). These mice could not be used to evaluate whether CCR7 deficiency in migDCs leads to increased immunity because ZBTB46 affects both tissue-resident and lymphoid-resident cDCs. Therefore, CCR7-dependent migratory DCs are not required for immunity.

ZBTB46-dependent cDCs are required for priming

To further clarify the cellular requirements for s.c. priming, we made BM chimeras comparing chimerization of control (CD45.1) BM to BM from several different DTR-expressing lines after DT treatment to deplete distinct cell populations. Chimerization was needed to avoid toxicity associated with DT administration directly to CD11c-DTR mice, although irradiated chimera recipients are older and generally have
A significant reduction in clonal expansion was observed after 4 d of culture in Z-DTR and in CD11c-DTR compared with control chimeras and littermate chimeras that had not received DT (Fig. 6C, blue). In L-DTR chimeras, although radioresistant populations such as LCs were spared, radiosensitive Langerin+ CD103+ migDCs were deleted. Consistent with the observation that L-DTR unchimerized mice had heightened immunity, depletion of Langerin+ CD103+ DCs.
in L-DTR chimeras produces heightened ex vivo CD4+ immune responses when compared with CD45.1→CD45.2 control chimeras.

Z-DTR does not delete monophasyotic lineages or PDCs and immunity was lost in Z-DTR mice, suggesting these subsets are not requisite. To confirm that CD11bhigh monocytes,
monocyte-derived DCs, and macrophages were not required for immunization, we examined the CD11b-DTR mouse versus wild-type. We observed no significant difference in priming between groups after DT administration, further excluding the contribution of these cells (Fig. 6, D–F) and controlling for the effects of DT administration. These data suggest Zbtb46- and Flt3L-dependent cDCs mediate αCD205gag-p24 priming.

migDCs closely cluster and share expression of immunedampening genes, and pathways, across species

We were intrigued by the surprising yet consistent observation that deletion of one or multiple subsets of Langerin+ migratory DCs seemed to enhance immunity. Also, collective blockade of skin DC migration enhanced the immune response to priming in vivo; this appeared to occur irrespective of the presence of Flt3L, suggesting migratory DCs might be acting in concert to suppress immunity. We observed close principal component analysis clustering of migDCs to each other when compared with cross-presenting cDCs, monocytes or monocyte-derived DCs, or macrophages (Fig. 7 A). We observed similar clustering of skin-resident DCs in humans when compared with their developmental counterparts in blood (Fig. 7 B). To further investigate this, we sorted three subsets of migDCs and CD8α+ cDCs from the skin-draining LNs from Flt3L-treated mice, seeking transcripts commonly expressed across three migDC subsets at twofold differential expression when compared with LN-resident CD8α+ DCs. We identified common up-regulation of genes associated with immune suppression across all skin DC subsets when compared with lymphoid-resident cross-presenting DCs mouse (Fig. 7 C). To determine if this occurs in people, we also analyzed transcripts from three subsets of human migDCs isolated from skin versus blood BDCA3+ cross-presenting DCs that were available in the NCBI Gene Expression Omnibus (GEO; see Materials and methods; Haniffa et al., 2012). We observed overlap in the genes expressed in the skin-resident or skin-migrated DCs across both species (Fig. 7 C). We performed our study in Flt3L-treated mice. Prior work has demonstrated immune dampening genes in a murine CD103+ migratory lung DC subset when compared

Figure 6. ZBTB46-dependent classical DCs are required for s.c. protein immunization.

(A–C) CD45.2 mice were irradiated and reconstituted with bone marrow as indicated. Chimeras received DT on day −3 and −1 to prime and boost immunization with 0.5 µg αCD205gag-p24. Intracellular cytokine staining immediately ex vivo for (A) Spleen and (B) LN. Black shows staining for p17 peptide control (plus DT) versus red or blue (plus DT) p24 peptide challenge. (C) CFSE dilution of splenocytes after 4 d in culture. (A–C) Pooled from 4–5 independent experiments (n = 3–5 mice per group, based on survival after DT administration). No DT controls (blue) performed once across all 4 groups (n = 4–5 mice per group). Error bars show mean ± SEM. *, P ≤ 0.1; **, P ≤ 0.05; ***, P ≤ 0.01; ****, P ≤ 0.001. (D–F) CD11b DTR versus B6 control mice were administered DT on day −3 and −1 to prime-boost immunization with 0.5 µg αCD205gag-p24. CFSE dilution of splenocytes after 4 d in culture (D) and intracellular cytokine stainings (E–F). Black shows staining for p17 peptide control (plus DT), red or blue (plus DT) p24 peptide challenge. (D) CFSE dilution of splenocytes after 4 d in culture (D) and intracellular cytokine stainings (E–F). Black shows staining for p17 peptide control (plus DT), red or blue (plus DT) p24 peptide challenge. One representative experiment of n = 4–5 mice per group. Error bars show mean ± SEM. *, P ≤ 0.05; **, P ≤ 0.001; ***, P ≤ 0.001.
Figure 7. Migratory DC subsets cluster together and share higher expression of immune dampening genes and down-regulation of genes associated with DC activation when compared with cross-presenting DCs in mouse and human. (A) PCA on twofold change (relative to LN CD8\(^+\) cDCs) or greater in the top 15% of all genes (\(n = 1,290\)) genes for 3 individual migratory DC, versus LPS-treated CD205\(^+\) cDCs, DC-SIGN\(^+\) monocyte-derived DCs, and macrophages (mouse); \(n = 3\) or 4 independent sorting experiments and sample replicates. (B) PCA on twofold change (relative to BDCA3\(^+\) or BDCA1\(^+\) blood DCs) or greater in the top 15% of all genes (\(n = 920\)) for human skin-resident versus blood DC and monocyte subsets. (C) Genes shared across all three migDC subsets with twofold or greater difference compared with CD8\(^+\) cDCs (mouse) or blood BDCA3\(^+\) DC humans: (left) mouse and (right) human skin DC subsets. * denotes the fold difference was under the threshold cut off of twofold in at least one of three migDC subsets. (D) Cross-species IPA. 227 genes commonly up-regulated by twofold or greater across all 3 migratory skin DC subsets compared with classical cross-presenting DCs in both mouse and human. Red represents up-regulated pathways; green represents down-regulated pathways.
with CD8α+ lung LN DCs (Miller et al., 2012). For additional validation, we compared our dataset (Illumina) to additional datasets from skin DC subsets and LN CD8α+ cDCs available in the GEO (see Materials and methods), which were performed by an independent consortium on a different array platform (Affymetrix) and in the absence of Flt3L treatment (Fig. S3). We observed the same trend, which was unaltered by Flt3L treatment. Murine transcripts included SOCS2, PIAS3, PD-L1, ITGB8, and Spred1, CD63, IL4i1, IL15Ra, Tmem176A, and Tmem176B. ITGB8 induces the immunosuppressive cytokine TGFβ (Travis et al., 2007). Knockdown of the tetraspanin CD63 in APC enhances T cell function (Petersen et al., 2011), Tmem176A and 176B binding partners inhibit DC maturation (Louvet et al., 2005; Condamine et al., 2010). The immature state of DCs is associated with silencing of antigen-specific T cell responses, induction of T regulatory cells, and tolerance to self (Steinman et al., 2003; Ohnmacht et al., 2009). CCL5 is involved in DC migration (Sallusto et al., 2000), and tolerance to self (Steinman et al., 2003; Ohnmacht et al., 2011). Tmem176A and 176B binding partners inhibit tetraspanin CD63 in APC enhances T cell function (Petersen et al., 2000).

**DISCUSSION**

s.c. immunization is one of the most potent and common forms of immunization. Nevertheless, it remains incompletely understood in vivo, limiting the design and development of clinical agents to enhance immunity. To elucidate the role of different DC subsets in immune priming, we simultaneously examined development, antigen capture, gene expression profiling, and functional analysis of different DC subsets. We identified Flt3, ZBT46-dependent classical DCs as central to T cell priming to HIV gag-p24. Strikingly, despite enhanced antigen capture and Flt3L dependence, migDCs, including Langerin+CD103+ DCs, dampen the immune response in vivo and share common gene signatures related to blockade of DC and T cell activation. Our data reveal Flt3 signaling is central to productive immunity via classical DCs and suggest Flt3L may have therapeutic potential when coupled to DC-targeted and soluble protein vaccines, as Flt3L enhanced immunity in multiple settings.

During immunization, changes in the distribution and composition of DCs occur. LN-resident and migDCs redistribute their numbers in the skin-draining LN, a change largely accounted for by an increase in the population of Langerin+ dermal DCs (Kastenmüller et al., 2011). Although tightly regulated in the steady state (Shadle et al., 1989; Langley et al., 1993), we detect Flt3L immediately after s.c. delivery of protein immunization and note Flt3L administration alters the composition of cDCs and migDCs in the skin-draining LNs. Flt3 receptor signaling is also necessary for productive immunity. Our data therefore suggest Flt3–Flt3L interactions are likely central to changes in the composition of the LN DC compartment after immunization and requisite for immunity. As previously observed in spleen (Bozzacco et al., 2010; Sathe et al., 2011), we note that Flt3L expands PDCs and cDCs in skin-draining LN, with bias to CD8α+ CD205+ cross-presenting cDCs (Fig. 3, A and B). Flt3L bias to LN-resident cross-presenting DCs and improved antigen capture by DCs are mechanisms that may support enhanced protein immunization. Indeed, newly formed CD24+ Flt3L-dependent precursors to CD8α+ spleen DCs induce stronger viral recall than CD8α+ DCs (Bedoui et al., 2009b). cDCs from Flt3L-treated mice demonstrate a higher percentage of in vivo antigen capture due to a higher fraction of CD8+ CD205+ DCs being represented (Fig. 3, B and C). This does not exclude the possibility Flt3L could alter lymphatic antigen delivery, but suggests differences in composition may be responsible for the observed effect in vivo.

Robust mucosal immunity is of particular importance to preventing the pathogenesis of certain lentiviral infections, such as HIV. Preferential depletion of CD4+ T cells has been noted in the intestinal lamina propria during acute HIV infection (Brenchley and Douek, 2008). Flt3L enhances both CD4+ and CD8+ mucosal immunity in peripheral sites, including lung parenchyma, small intestinal lamina propria, and skin (unpublished data). Enhanced immunity was versatile and occurred with different antigens, microbial mimics, soluble and DC-targeted protein immunization, and increased serum IgG, lymphoid, and mucosal T cell responses. Coupling Flt3L to both soluble and DC-targeted protein vaccines with appropriate adjuvant for cross-presenting cDCs will likely be of clinical benefit.

Given a requirement for FLK and Zbtb46 with exclusion of migDCs, pDCs, monocytes, and macrophages, we demonstrate that Flt3L–ZBTB46-dependent cDCs are central to s.c. priming. T cell priming via LN-resident CD24+ CD8α DCs is consistent with our observation that Flt3L improved...
immunization with either TLR 3/MDA5 (polyIC[LC]) and TLR 4 (GLA-SE) microbial mimics as adjuvant. Fli3L-dependent CD240+ DCs from the spleen and BM cultures express higher transcripts for TLR3 and some TLR4, whereas the CD11b+ subset expresses predominantly TLR4 and no TLR3 (Naik et al., 2005). The requirement for cDCs and not migDCs is not likely caused by TLR restriction because CD103+ dermal DCs express higher levels of TLR3 (Jelinké et al., 2011) but are dispensable for immunization with polyIC[LC]. Finally, our observations in CCR7−/− mice, also may suggest monocyte-derived DCs are not required in the LN during priming because they require CCR7 for LN entry (Cheong et al., 2010). Although GLA-SE is a microbial mimic of LPS, and LPS induces monocyte-derived DCs, we observe that GLA-SE may act as adjuvant independently of monocyte-derived DCs in the skin-draining LNs. This was further validated using CD11b DTR mice in which deletion of monocytes and monocyte-derived DCs, but not CD8α+ CD205+ cDCs, occurs (Choi et al., 2011), which did not impair CD4+ IFN-γ responses.

Our data support a functional and genetically programmed role for migDCs in dampening immunity. Current vaccine design efforts are centered on targeting antigen to skin DCs and may inadvertently trigger a program of immune suppression. At high and limiting dose aCD205-gag p24, we do not observe a requirement Langerin+ DCs in s.c. immunization. Rather we observe enhanced T and B cell immunity. Enhanced immunity to s.c. and i.d. antigen are observed in the complete absence of skin DC migration to the draining LNs. Consistent with a role in dampening immunity in vivo, three individual skin DC subsets share common expression of genes associated with tolerance when compared with lymphoid and blood cross-presenting DCs. These included common programs shared across species that block DC migration and activation, and those involved in direct inhibition of T cell activation, such as PD-L1. Thus, the immune response likely results from a complex interplay of tolerizing and immunizing DC activity in LNs. The cues that dictate when migDCs are poised to induce tolerance versus immunity to self and nonself-antigens remain to be further investigated. These cues may be conferred by the cutaneous or peripheral microenvironment and likely extend beyond hematopoietic lineage specification. Our study suggests Langerin+CD103+ DCs from tissue directly oppose the activity CD8α+ cDCs in LNs in vivo despite common origin, shared developmental pathways, and irrespective of developmental potentiation with Fli3L. Indeed, we find migDCs from both Fli3L-treated and untreated mice share elevated expression of immunodampening gene signatures and pathways (Fig. 7 and Fig. S3).

In contrast to a deterministic model in which distinct populations of DCs are developmentally programmed, the role of different DC subsets in priming and tolerance could relate to their spatial and temporal distribution in the LN, tissue residence, and antigen access. cDCs lie close to the LN reticular system, or conduits, where they rapidly uptake and present soluble antigen from lymph (Sixt et al., 2005). migDC subsets carry high concentrations of processed antigens to the LN inner and outer paracortex near HEVs (Bajénoff et al., 2003; Kissempfennig et al., 2005) and arrive in the LN later after immunization (Itano et al., 2003). That we observe enhanced antigen capture by migDCs in LNs is consistent with prior observations of soluble antigen pE–I–Ab complexes detected on skin-derived DCs already present in the LNs as early as 30 min after s.c. antigen injection (Itano et al., 2003). Based on the early kinetics of peptide complex detection, migDCs in LN were thought to initiate T cell priming. However, when the injection site is removed 5 h after s.c. priming, preventing the arrival of additional migDCs from periphery to LN, clonal proliferation is not impaired, suggesting redundancy of migDCs that acquired antigens in the periphery and traffic into the LN. However, injection site removal studies cannot exclude the contribution of preexisting LN-resident migDCs (Itano et al., 2003). Rather, when we tested CCR7−/− mice where migDCs are entirely absent from the LN at the time of priming, we observe enhanced but not diminished in vivo immune priming supporting a role for migDCs at the LN in dampening immunity and further supported by their common transcriptomics program.

Previous studies addressing DC subset contributions have largely examined the CD4+ T cells response to s.c. OVA immunization (Allenspach et al., 2008). In this system, immune responses depended on radio-resistant skin DCs (i.e., mainly LCs) in the draining LNs. Differences in our observations from such work may result from several notable differences in the model systems used, including the antigen (HIV gag-p24 vs. OVA), the adjuvant (microbial mimic vs. complete or incomplete Freund’s adjuvant), deliver strategy (DC-targeted vs. non-targeted), differences in the naive polyclonal repertoire in our study versus intravenous adoptive transfer of a single-affinity clone (OT-II), and the model system used to test the contribution of migDCs (restricted migration and complete absence in the LN in CCR7−/− or DTR depletion [our study] versus normal migration capacity of migDCs but with impaired MHCII presentation [Allenspach et al., 2008]). Additional work using OT-II T cell transfer and immunization with chemically coupled αCD205-OVA in the CCR7−/− versus control mice observed a diminished T cell response in CCR7−/− mice (Ohl et al., 2004). Therefore, the difference we observe compared with previous studies with OVA and OT-II is most likely related to the nature of the antigen and uniform T cell repertoire in the setting of transgenic models rather than antigen delivery (DC targeting), adjuvant, and the model system used to distinguish migDCs from LN-resident DCs. Indeed, in prior experiments when the concentration of OVA was increased the requirement for migDCs was relieved (Allenspach et al., 2008). Thus, dependence for the migDCs in the OVA transgenic system may relate to specifics of that system, such as affinity of the clone, low-dose antigen, the number of peptide MHCII complexes, the distribution of intravenously transferred transgenic T cells, and competition of naïve transgenic cells for antigen. Consistently, migDCs failed to induce delayed proliferation of OT-II cells in the absence
of lymphoid-resident DC antigen presentation (Allenspach et al., 2008), supporting our observation LN-resident DCs are required to initiate immunity and that lymphoid-resident DCs selectively trap antigen-specific lymphocytes in the draining LN (Itano et al., 2003). Our initial observation that migratory DCs dampen the immune response to high- and low-dose antigen was validated with four selective DC ablation models and transcriptome analyses.

Lastly, we note that CCR7 is dispensable on both T cells and DCs for productive immunity. Though migDCs are entirely CCR7-dependent, naive T cell entry into LN is partially dependent on CCR7 and reduced to 30–50% of wild-type levels in peripheral LNs (Forster et al., 1999). T cells can be classified as naïve central memory (Tcm) CD45RA–CCR7–, effector memory (Tem) CD45RA–CCR7+ or terminally differentiated effector cells (TemRA) CD45RA+CCL7– (Sallusto et al., 1999; Sallusto and Lanzavecchia, 2009; Pepper et al., 2010). That CCR7 dependence was not observed on T cells may relate to the observation in vaccinia virus and yellow fever vaccines that CCR7 dependence was not observed on T cells and exhibit strong proliferation in vitro, suggesting a lack of terminal differentiation (Ahmed and Akondy, 2011).

Advances in immunotherapies to block immunosuppression include CTLA-4 and PD-L1 blockade, and dramatically expand the therapeutic window for immune priming. Protein immunization offers a safe and efficacious strategy. We demonstrate the Flt3 pathway is central to protein immunization and can be exploited to potentiate immunity via cDCs. Our data further reveal that a robust T cell and humoral immunity to protein immunization with clinically relevant vaccines, and with a polyclonal T cell repertoire, is centered on ZBTB46+/−and Flt3L-dependent cDCs. We establish skin-derived migDCs, including Langerin+103+ DCs, are not required at the LN for s.c. protein vaccination immunization. Instead these populations collectively and individually dampen immunity and share common expression of genes associated with immune inhibition. Though Langerin+CD103+ DCs of the skin share developmental origin with CD8α+ cDCs in lymphoid tissue, they may be conditioned by tissue microenvironment to inhibit DC and T cell activation. This cannot be overcome by enhancing their numbers, antigen capture, or developmental conditioning. Thus, future immunization strategies for the clinic will couple likely protein immunization and Flt3L treatment to target antigen to, and simultaneously potentiate the development of and antigen capture by, LN-resident cDCs.

MATERIALS AND METHODS

Mice, chimerization, and deletion. C57BL/6 mice (B6) were purchased from Taconic Labs or bred at The Rockefeller University. The Langerin-GFP mice were generously provided by B. Malissen, bred as homozygotes at The Rockefeller University; and have been previously described (Kuesenburger et al., 2008). BM chimeras used 8–9 wk-old irradiated recipients (900 cGy plus 550 cGy) with 3 h between irradiations; cell suspensions of BM were injected intravenously immediately after (3 × 107 BM cells transferred per recipient). Mice were maintained after radiation on antibiotic supplemented food (TestDiet) for 12 wk before use. All mice were housed in specific pathogen-free conditions. Protocols were approved by the Rockefeller University Animal Care and Use Committee.

Tissue harvest and cell preparation of DC. 6–8 wk-old C57BL/6 F1 or Langerin-GFP mice were injected s.c. to the flank with the endotoxin-free (<0.0064 EU/mg), GMP grade, recombinant human Flt3L (Cell笛ex) at 10 µg/mouse/day diluted in sterile PBS for 10–14 d. For sorting skin DC subsets, Flt3L-treated Langerin GFP mice were used to obtain sufficient numbers of migratory DCs from the skin-draining LNs. Skin-draining LN and spleen was isolated from individual mice, teased, or ballooned, and incubated for 25 min at 37°C in Collagenase D (400 U/ml Roche) in Hanks’ Balanced Salt Buffer (Invitrogen). After incubation, 0.5 M EDTA was added to a final concentration of 10 mM EDTA for disruption of DC–T cell complexes and the sample was incubated for an additional 5 min at 37°C. For spleen cell preparation, ACK lysis of red cells was performed. Undigested fibrous material was filtered through a 70-µm cell strainer. Subsequent washes were performed with ice-cold PBS with 2% FCS. For crawl-out assay, individual ears were harvested, washed in 70% EtOH and split dermal side down into complete RPMI media. At 72 h, cell suspensions were isolated and filtered. The pellet was washed twice and incubated in Fc block with 2% rat serum before cell surface marker antibody staining.

After size, live/dead, and exclusion criteria (CD3, CD19, NK1.1 exclusion), DC subsets were sorted as described (Fig. 3 and Fig. S2). RNA was prepared by standard methods using TRIzol (Invitrogen) and further purified using RNeasy MiniElute clean up (QiAGEN). Purify analysis was done by nanodrop and Eukaryote Total RNA Pico Series II (Agilent). rRNA was amplified and hybridized on the Illumina MouseRef-8 v2.0 Expression BeadChip.

Vaccination protocol. Age, gender-matched control, and experimental mice were injected daily during both the prime and boost phase by i.p. injection with Flt3L, as above, in sterile PBS, or by PBS alone (control) for 10 d. At day 8, αCD205-gag p24 or αCD205-OVA was injected s.c. into the footpad or by i.p. route, as indicated with 20 µg of GLA-SE (ImmuGene Design) or 25 µg of polyIC-stimulated with poly-L-lysine (poly ICl[c]; Loungh et al., 2009).

Boost vaccination was administered exactly 4 wk later. For s.c. priming to αCD205-gag p24, mice in all groups were sacrificed 7 d after immunization. For s.c. priming to αCD205-OVA, mice were sacrificed at day 11 after boost for IP priming at 21 d. In mice vaccinated to αCD205-gag p24, peptide challenge with p24 pools or p17 (control) pools at 1 µg/ml for intracellular cytokine staining with 2 µg/ml anti-CD28 antibody was added, and at 0.05 µg/ml for CFSE 4 d cultures. For mice vaccinated to αCD205-OVA, O1-I and O1-II peptides were pooled versus p24 (control) at 1 µg/ml for ICS with 2 µg/ml anti-CD28 antibody, and for CFSE cultures at 0.05 µg/ml.

T cell isolation for proliferation assay or intracellular cytokine stain (spleen, lungs, and LN). Individual vaccinated mice were sacrificed, and serum collected by cardiac bleed with separation of serum from heme by centrifugation at 13,000 rpm in BD microtainer serum separator tubes (ref. 365956) in a table-top rotor (Eppendorf 5417R) at 4°C. Intracardiac perfusion was performed with PBS into the right ventricle to balloon the lungs and flush leukocytes and RBCs from the pulmonary circulation. Individual lung lobes were diced, taking care to avoid the mediastinal LN, balloononed with Collagenase D, and then further dissociated with pressure using the end of a 5-ml syringe, followed by incubation at 37°C for 20 min. 10 mM EDTA was added for the last 5 min of incubation. ACK lysis was performed as above. Spleens and LN were harvested in RPMI complete media with 5% FCS, mashed between 2 sterile glass slides, washed with RPMI complete media, and then filtered through a 70-µm filter. Spleen samples but not LNs went...
through subsequent ACK lysis as described, and were washed twice and counted. As described by Trumpfthaler et al. (2008) 500K splenocytes were incubated for 6 h at 37°C with p24 pooled peptides or p17 pooled peptides at a final concentration of 1 µg/ml with 2 µg/ml anti-CD28 antibody (clone 37.51; American Type Culture Collection). After 1-h incubation, Brefeldin A was added to a final concentration of 10 µg/ml for the remaining 5 h to allow intracellular cytokine accumulation. For all subsequent steps, cells were washed and stained in ice-cold PBS with 2% FCS. For proliferation assays, splenocytes were labeled with 1 µM/ml CFSE (Sigma-Aldrich) and plated with 0.05 µg/ml p24 or p17 peptide pool. At 72 h, supernatants were harvested for cytokine ELISA. At 96 h, cells were isolated, washed, and intracellular cytokine staining was performed as previously described.

**Serum gag-specifc IgG and Flt3L ELISA.** Serum was prepared by cardiac bleed, followed by serum separation using microtainer tubes (363956; BD). Serum gag-specific ELISA was performed by coating NUNC plates (Apogen 80040 LE 0903) with 2 µg/ml of gag p24 recombinant protein in PBS or ELISA coating buffer overnight at 4°C. Plates were washed 3–5 times with PBS and blocked with blocking solution (1X PBS, 5% goat serum, and 0.1% Tween-20) for 1 h at 37°C, followed by sample addition at serial 10-fold dilution (dilutions made into blocking solution) for 2 h at 25°C, followed by 3–5 subsequent washes. A 1:10,000 dilution of goat anti-mouse IgG HRP (Jackson Immunoresearch Laboratories; 115–035-071) was added according to standard protocols for 1 h at 37°C, the plate was washed 3–5 times before addition of 100 µl 1X TMB Substrate Solution (eBioscience; 00–4201–56), stopped with addition of 2N sulfuric acid, and read at 450/470 nm. Murine Flt3L ELISA was performed on serum isolated from 3–5 individual mice using the RD mFlt3L ELISA kit (E90038Mu).

**Lamina propria isolation.** To prepare single intestinal cell suspension, part of small bowel including jejunum and ileum or a large bowel (cecum and colon) were excised. Peyer’s patches were removed from the small intestinal part. Lamina propria isolation. The resulting cell suspension was layered on a 44%/66% Percoll (GE Healthcare) gradient and the interface was collected to obtain an enriched monocytic cell population. The results of replicates were averaged. Normalized data were filtered for gene encoding regulators with a coefficient of variation of less than 0.5 in population replicates. Individual dermal migratory DCs in LNs were compared against LN-resident CD8α+ DCs, and differentially expressed genes of twofold or more were assessed by variance across the DC populations. Microarray data is available macrophages, monocyte-derived LN DCs, LPS-treated classical DCs in LNs, and LNs of Flt3L-treated mice from GEO under accession no. GSE53888. Additional murine microarray files (Miller et al., 2012) were downloaded from GEO under the accession no. GSE15907 (subset data from accession nos. GMS538257–GMS538257 and GMS538268–GMS538279).

A custom chip technology for Affymetrix Mouse Gene 1.0 ST was created to import the raw data into GeneSpring NGS. Human microarray files (Hanna et al., 2012) were downloaded from NCBI Gene Expression Omnibus under the accession no. GSE35459 (subset data from accession nos. GSM688894–GSM688898, GSM688910–GSM688917, and GSM688922–GSM688925). A custom chip technology for Illumina HumanHT-12v4.0 expression BeadChip was created to import the raw data into GeneSpring NGS.

Raw files were preprocessed and normalized using GeneSpring GX Version 12.5 RMA algorithm. Resultant data were pooled into groups by various subtypes. The results of replicates were averaged. Normalized data were filtered for gene encoding regulators with a coefficient of variation of less than 0.5 in population replicates. Individual dermal migratory DCs in LNs were compared against LN-resident CD8α+ DCs, and differentially expressed genes of twofold or more were assessed by variance across the DC populations with the one-way (ANOVA), corrected for multiple hypotheses testing with Benjamini and Hochberg FDR of ≤0.05.

**Statistical analysis.** For data reported in vaccine experiments, error bars represent the standard error of the mean plotted between 3–5 individual animals per experiment per replicate. Statistical analysis for ICS and CFSE was performed using the Mann-Whitney test between two groups was done using Prism software (P ≤ 0.05, not significant [ns], * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001). For serum Flt3L ELISA detection (Mann-Whitney test) and DC expansion (unpaired Student’s t test), error bars represent the SD between three to five individual mice per group. For serum gag-p24 or OVA IgG detection, error bars represent the mean ± SEM and were analyzed using an unpaired Student’s t test.

**Online supplemental material.** Fig. S1 shows the gating schema for intracellular cytokine staining assays. Fig. S2 shows the gating of LN-resident DC subsets. Fig. S3 depicts fold change in individual migratory skin DC subsets from our Flt3L-treated mice on the Illumina platform, versus Imgen consortium data on untreated mice on the Affymetrix platform. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20131397/DC1.

We are deeply grateful to Ralph M. Steinman for his mentorship, support, and wisdom. The authors thank Christine Trumpfthaler for advice with cd205-HIV gag immunization, Tibor Keler and Celldex therapeutics for use of recombinant Flt3L, Immune Design Corporation for use of GLA, Judy Adams for assistance with preparation of the figures, Jennifer Ogene, Anthony Rodriguez, and Sandy King for technical assistance, and Marguerite Nulty for administrative assistance. We thank Michelle Lowes and Andrew Sikora for critical reading of the manuscript.
REFERENCES


SUPPLEMENTAL MATERIAL

Anandasabapathy et al., http://www.jem.org/cgi/content/full/jem.20131397/DC1

Figure S1. Gating schema for CD4⁺ and CD8⁺ T cell intracellular cytokine staining after 6 h peptide restimulation.

Figure S2. Skin draining LN gating scheme. (A) Skin-draining LN DC gating schema pDCs, cDCs, and mDCs based on CD11c and MHC I-A/I-E mean fluorescence intensity from representative Flt3L-treated animal. (B) Langerin intermediate (green) and high population (red) staining versus rat IgG2a isotype control (gray) by intracellular staining. CD103 staining (orange) versus Armenian Hamster Isotype control (gray), aCD205 staining (purple) versus rat IgG2a Isotype control (gray).
Figure S3. LN-resident migDCs share common immune dampening gene signatures and down-regulation of some activation pathways. Comparative analysis of Illumina array (Flt3L treated) against Affymetrix array (non-Flt3L treated, Immgen consortium). * denotes the fold difference was under twofold in at least one of the three migratory DC subsets in one of the two datasets.