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Metabolic control of cell fate bifurcations in a hematopoietic progenitor population

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Abstract

Growth signals drive hematopoietic progenitor cells to proliferate and branch into divergent cell fates, but how unequal outcomes arise from a common progenitor is not fully understood. We used steady-state analysis of *in vivo* hematopoiesis and Fms-related tyrosine kinase 3 ligand (Flt3L)-induced *in vitro* differentiation of dendritic cells (DCs) to determine how growth signals regulate lineage bias. We found that Flt3L signaling induced anabolic activation and proliferation of DC progenitors, which was associated with DC differentiation. Perturbation of processes associated with quiescence and catabolism, including AMP-activated protein kinase (AMPK) signaling, fatty acid oxidation, or mitochondrial clearance increased development of cDC2 cells at the expense of cDC1 cells. Conversely, scavenging anabolism-associated reactive oxygen species skewed differentiation towards cDC1 cells. Sibling daughter cells of dividing DC progenitors exhibited unequal expression of the transcription factor interferon regulatory factor 8 (IRF8), which correlated with clonal divergence in FoxO3a signaling and population-level bifurcation of cell fate. We propose that unequal transmission of growth signals during cell division might support fate branches during proliferative expansion of progenitors.

Keywords

Flt3L; asymmetric division; PI3K; AMPK; dendritic cell

INTRODUCTION

Hematopoietic progenitor cells undergo regulated differentiation to populate the mature blood lineages. During cellular differentiation, multi-potent progenitor cells undergo progressive specification and bifurcate into divergent lineages. Dendritic cells (DCs) are

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CONFLICT OF INTEREST

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sentinel immune cells that develop from hematopoietic progenitors into functionally distinct subsets, including two types of conventional DCs (cDCs), cDC1 and cDC2, and plasmacytoid dendritic cells (pDC).¹ Fms-related tyrosine kinase 3 ligand (Flt3L) induces DC differentiation^{1,2} and activates nutrient-sensitive signaling pathways, including phosphoinositide 3-kinase (PI3K)/mammalian target of rapamycin (mTOR).^{3–7} Emerging evidence implicates the metabolic state of hematopoietic cells as a determinant of cell fate.⁸ Here we examined the role of selective metabolic processes in DC progenitor cell differentiation. Our findings are consistent with a model wherein activated progenitors undergo anabolic induction that drives cellular expansion. Inequality in the transmission of anabolic signaling intensity may support the production of a diverse rather than monotonic set of cellular descendants.

RESULTS AND DISCUSSION

Cellular anabolism supporting DC progenitor division and differentiation

As predicted², Flt3L induced hematopoietic progenitor cell proliferation and differentiation of CD11c+ cDC (MHCII+ B220-) and pDC (MHCII-lo/int B220+) subsets after multiple divisions (Figure 1a). Reciprocal expression of the transcription factors interferon regulatory factor 8 (IRF8) and interferon regulatory factor 4 (IRF4) among cDCs was evident (Figure 1b), with IRF8-high and IRF4-high cells phenotypically resembling cDC1 (CD24+) and cDC2 subsets (Sirp1- α + CD11b+), respectively.

Receptor tyrosine kinases can induce nutrient uptake and aerobic glycolysis (Warburg effect) to support rapid cell division and anabolism.⁹ Proliferating, Flt3L-stimulated progenitor cells exhibited an increase in cell size, enhanced expression of nutrient transporters, CD71, CD98, Glut1 (Figure 1c), and increased glucose uptake (Figure 1d) compared to the undivided cells. We then used the inhibitor 2-DG to inhibit anabolism-associated aerobic glycolysis. Treatment with 2-DG blocked Flt3L-induced proliferation in a dose-dependent fashion, indicating that inhibition of aerobic glycolysis and/or induction of stress pathways secondary to this metabolic perturbation could alter DC development (Figure 1d). Flt3 receptor tyrosine kinase signaling to anabolic cellular metabolism thus initiates a program of division-linked DC differentiation.

Catabolism-associated processes impart DC fate branching

Among cDCs, we found that IRF4+ cDC2 cells expressed greater levels of CD71 than cDC1 cells (Figure 1c), suggesting the possibility of unequal metabolism during lineage specification. In lymphocytes, cellular anabolism is characterized by PI3K/AKT/mTOR signaling, excess nutrient uptake, aerobic glycolysis, macromolecular biosynthesis, inhibition of autophagy, and production of reactive oxygen species (ROS), cellular catabolism is characterized by AMPK signaling, low nutrient flux, autophagic self-digestion, fatty acid oxidation, oxidative phosphorylation, and enhanced mitochondrial turnover.^{9,10} Inhibition of catabolism-associated fatty acid oxidation did not affect total cDC/pDC development, but significantly increased the frequency of IRF4+ cDC2 cells and decreased the frequency of IRF8+ cDC1 cells (Figure 2a). While cellular anabolism apparently drives

division of progenitors, cellular catabolic mechanisms might divert cells into alternative differentiation fates.

Anabolism and catabolism have opposing effects on mitochondrial dynamics, and PI3K/mTOR activity inhibits mitochondrial autophagy (mitophagy). We used a fluorescence-based assay of mitochondrial clearance (mitoclearance¹¹) to follow the elimination of aged mitochondria in dividing progenitor cells. Prior to Flt3L stimulation cells were pulse labeled with Mitotracker Green and washed free of excess dye. After each division, fluorescence is lost due to passive apportioning of old mitochondria between daughter cells¹¹ (Figure 2b). A component of mitoclearance also results from active elimination insofar as the rate of clearance is augmented or delayed by stimulants or inhibitors of general autophagy, respectively.¹¹ Old mitochondria in the DC progenitors are also being actively degraded, as demonstrated by their diminished clearance during inhibition of mitophagy using mDivi-1 to prevent mitochondrial fission¹¹ (Figure 2b). We found that the clearance of aged mitochondria was enriched among cells with higher levels of IRF8 expression (Figure 2c). Mature IRF8+ cDC1 cells ultimately have normal or greater total mitochondrial content as well as mitochondrial membrane potential compared to cDC2 cells (Figure 2d). However, cells with greater clearance of aged mitochondria acutely appear to have slightly lower total mitochondrial content than cells with mitochondrial stasis (Figure 2e). Treatment with m-Divi1 (to inhibit mitochondrial fission) or M1 (to promote mitochondrial fusion¹²) had minor effects on overall cDC/pDC development, but each perturbation significantly promoted the frequency of IRF4+ cDC2 over IRF8+ cDC1 differentiation (Figure 2f). Clearance of old mitochondria therefore appears to be important for balanced DC subset specification.

Mitochondrial ROS (mROS) mediate many biological effects of cellular anabolism, and we found that mROS production is higher in cells with less clearance of aged mitochondria (Figure 2g).¹¹ When we examined total mROS levels, we found that Flt3L-stimulated progenitors undergoing proliferation had bimodal mROS production, which inversely correlated with IRF8 expression (Figure 2g). Scavenging global ROS with N-acetyl cysteine (N-Ac) increased the frequency of IRF8+ cDC1 over IRF4+ cDC2 differentiation, without affecting cDC/pDC development (Figure 2h). Taken together, together these results suggest that Flt3L-induced DC differentiation is characterized by heterogeneity in the degree of anabolic metabolism, which may contribute to cell fate divergence.

Catabolism-associated AMPK- α 1 activity promotes cDC1 differentiation

Despite undergoing anabolism-associated proliferation, dividing progenitors exhibited evidence of catabolism-associated AMPK signaling by phospho-flow cytometry, including phosphorylation of AMPK itself as well as the AMPK targets acetyl-CoA carboxylase (ACC) and Raptor (Figure 3a). We then determined whether AMPK- α 1, an important regulator of hematopoietic stem cell metabolism¹³, is important for subset-specific DC differentiation. AMPK- α 1 deficiency did not significantly affect the ratios of cDC and pDC *in vitro* (Figure 3b) but did, however, skew the frequencies of cDC differentiation towards the IRF4+ cDC2 fate. Combining perturbations of catabolism-associated AMPK signaling and mitoclearance compounds the switch to anabolism-associated cell fate in lymphocytes.¹¹

Likewise, we found that inhibition of mitochondrial fission with mDivi-1 compounded the phenotype of AMPK- α 1 deficient cells, worsening the defect in IRF8+ cDC1 differentiation (Figure 3b). While no differences in bone marrow DC progenitor frequencies were observed, we found that AMPK- α 1 was also required for efficient terminal cDC1 differentiation *in vivo* (Figure 3c). In skin-draining lymph nodes, proportions of cDCs and pDCs were unaffected by loss of AMPK- α 1. Resident CD8 α + cDC1s were, however, significantly reduced in AMPK- α 1 deficient animals, alongside a more minor reduction in migratory CD8 α - CD11b- DCs. Conversely, we observed an increased frequency of CD11b+ cDC2 cells. Catabolic signaling mediated by AMPK- α 1 may be involved in DC progenitor cell fate bifurcations and balanced cDC1/cDC2 differentiation *in vitro* and *in vivo*.

Unequal expression of IRF8 in sibling DC progenitors

In Flt3L-stimulated progenitors, we found heterogeneous expression of IRF8 accompanied cell division and occurred even prior to DC differentiation (Figure 1a and Figure 4a). IRF4 induction, however, was found to be more monotonic and delayed, becoming evident only at late culture timepoints and in mature, terminally differentiated cells (Figure 4a). In other immune cell fate bifurcations (germinal center B cell versus plasma cell), IRF8 and IRF4 are reciprocally expressed and quantitatively antagonize each other's function and expression.^{14,15} Mechanistically, the outcome of the IRF8-versus-IRF4 competition is determined by the strength of PI3K signaling and inactivation (i.e. displacement from nucleus) of FoxO factors, leading to ascendance of IRF4 over IRF8.^{14–17} By performing cell sorting and replating experiments using cells from IRF8-eGFP reporter mice, we found that IRF4-negative, CD11c-negative DC progenitors with intermediate levels of IRF8 yielded a bifurcating, mature CD11c+ population composed of IRF8-hi cDC1 and IRF4+IRF8-lo cDC2 descendants (Figure 4b). By contrast, IRF4-negative, CD11c-negative DC progenitors with high levels of IRF8 produced only IRF8-hi cDC1 descendants.

At the clonal level, the IRF8-versus-IRF4 competition plays out as opposite outcomes during cell division because a progenitor transmits unequal PI3K activity to its daughter cells, manifest as both discordant FoxO inactivation¹⁷ and asymmetric PI3K enzymatic activity (indexed by PIP3 lipid staining). To determine whether the population-level bifurcation in cDCs arising from DC progenitors could be the result of intra-divisional, clonal bifurcation, we examined the expression of IRF8 to assess clonal disparity and the activity (localization) of FoxO as an index of PI3K intensity in sibling cell pairs.

Cells in interphase, metaphase, and anaphase did not exhibit evidence of polarized IRF8 subcellular localization (Figure 4c). In daughter cells undergoing cytokinesis, however, substantial inequality of IRF8 protein expression was readily detected. Akin to the low-level expression of IRF4 protein in progenitors (Figure 4a), the levels of IRF4 were undetectable by microscopy in conjoined sibling pairs. We also found that Flt3L signaling induces phosphorylation of FoxO3a, a factor which regulates DC development¹⁸ (Figure 4d). Amongst sibling cell pairs with unequal IRF8 abundance, FoxO3a nuclear versus cytoplasmic localization was discordant, with nuclear (active) FoxO3a protein predominantly found in the IRF8-high daughter cell.

IRF8-intermediate progenitors, which exhibit increased mitostasis and ROS production (Figure 2c and Figure 2g), appear to specifically harbor potential to produce IRF4+IRF8-lo cDC2 cells (Figure 4b). Together with the enhanced frequency of IR4+ cDC2 cells resulting from inhibited mitochondrial autophagy, the reduced frequency of IR4+ cDC2 cells resulting from scavenging ROS, and the results of other metabolic perturbations presented herein, our data support a model in which unmitigated anabolic signaling favors the IRF4+ cDC2 fate, while a component of catabolism may temper the signal strength and divert cells toward the IRF8+ cDC1 fate. Whether and how sibling progenitor cells might undergo unequal information transfer, such as asymmetric PI3K signaling, will require further investigation. The cell biological mechanism may relate to asymmetric clustering of Flt3 receptor-associated signaling events or another cell polarity mechanism.^{10,19} Nonetheless, the association between metabolism and hematopoietic cell fate bistability could open new avenues for therapeutic intervention.

METHODS

Mice

All animal work was conducted in accordance with the Institutional Animal Care and Use Guidelines of Columbia University. Mice were housed in specific-pathogen-free conditions. Both male and female mice between 6–8 weeks of age were used. C57BL/6 wild type, IRF8-eGFP reporter²⁰, and AMPK- α 1 knockout mice¹³ were used.

DC Differentiation Assay and Drug Treatments

BM progenitors were cultured in RPMI supplemented with 10% FBS, 1% penicillin/streptomycin, and recombinant Flt3L (PeproTech, Rocky Hill, NJ, USA) at 100ng/mL. Cells were labeled with CellTrace Violet, a cell proliferation dye (CTV, Thermo Fisher Scientific, Waltham, MA, USA). All drugs were added at the beginning of culture and included: mDivi-1 (Cayman Chemical, Ann Arbor, MI, USA, 10 μ M), M1 (Sigma-Aldrich, St. Louis, MO, USA, 5 μ M), N-Acetyl cysteine (N-Ac, Sigma, 5mM), 2-deoxyglucose (2-DG, Sigma, 100 μ M–1mM), and etomoxir (Cayman, 100 μ M), an inhibitor of the essential fatty acid oxidation enzyme, carnitine palmitoyltransferase I. All cultures were harvested for FACS analysis of DC subsets at day 9 of stimulation with Flt3L, unless otherwise specified.

Flow Cytometry and Immunofluorescence Microscopy

Flow cytometry and immunofluorescence (IF) microscopy were performed as previously described^{11,17}. Mitochondrial membrane potential was measured by labeling cells with tetramethylrhodamine ethyl ester (TMRE, Sigma). For mitochondrial clearance assays, cells were pulse labeled with Mitotracker Green before stimulation with Flt3L. For lineage potential experiments, IRF8-eGFP reporter cells were labeled with proliferation dye and then FACS sorted at day 4–5 of culture with Flt3L into CD11c- IRF8-high and IRF8-int populations. Sorted cells were then cultured for an additional 4 days in Flt3L-supplemented media. Mitotic cells were identified by tubulin morphology, and random cells throughout the field were imaged. To quantify asymmetry by microscopy, mitotic cells were randomly selected and imaged throughout the field of view. Fluorescent signal was thresholded to remove background, and the integrated fluorescence density in each nascent daughter cell or

half of the metaphase plate was calculated. Asymmetry was calculated as (fluorescence daughter 1 – fluorescence daughter 2) / (fluorescence daughter 1 + fluorescence daughter 2), with values over 0.2 considered asymmetric; dashed blue lines on asymmetry plots denote this asymmetry cutoff.

Statistical analyses

Statistical analyses were performed using GraphPad Prism (Version 6, La Jolla, CA, USA). P-values and significance cut-offs are specified in each figure legend.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Cytokine-induced DC developmental branching accompanying progenitor cell expansion.

(a) Expression of CD11c versus cell division at day 3, 6, and 9 of culture. Right: cDC (CD11c+ MHCII+) and pDC (CD11c+ B220+ MHCII-) subsets within CD11c+ cells at day 9. Statistic indicates mean +/- standard error the mean (SEM) for gate. (b) Left, reciprocal expression of IRF8 and IRF4 marks distinct cDC1 and cDC2 subsets within cDCs. Statistic indicates mean +/- SEM for gate. Right, surface phenotype (CD24, Sirp1- α , and CD11b) expression) of cDC1-like (IRF8+) and cDC2-like (IRF4+) subsets. Histograms are representative of at least 3 independent experiments. (c) FIt3L stimulation increases anabolic

signaling in DC progenitors. Left panels, representative histograms and quantification of forward scatter (FSC), an index of cell size, and nutrient transporter expression (CD98, CD71, and Glut1) by proliferating (grey fill) and undivided (black line) progenitors at day 3 of culture. $*P < 0.05$. $**P < 0.01$. $***P < 0.001$. Student's *t*-test. Upper right panel, representative histogram of CTV dye dilution for gating of undivided and proliferating progenitor cells at day 3 of culture. Lower right panel, expression of CD71 by mature IRF8+ (black line) and IRF4+ (grey fill) cDCs at day 9 of culture. Histograms are representative of 2 independent experiments. (d) Glycolysis is required for Flt3L-induced DC progenitor cell proliferation. Left, increased glucose uptake in proliferating DC progenitors at day 3 of culture. $**P < 0.01$. Student's *t*-test. Right, cell division with increasing concentrations of glycolysis inhibitor 2-DG at day 9 of culture. Plots are representative of 3 independent experiments.

Figure 2.

Mitochondrial dynamics and metabolism promote selective DC differentiation. (a) Fatty acid oxidation modulates cDC subset development. Left, frequency of cDC and pDCs following etomoxir treatment. n.s. not significant. Student's *t*-test with Holm-Sidak correction. Right, development of IRF8+ and IRF4+ DC subsets following etomoxir treatment. ***P* < 0.01. Paired *t*-test. (b) Mitoclearance assay. Progenitors were pulse-labeled with MTGreen at day 0 or day 5 of culture and assessed for decay of fluorescence 3 days later. FACS plots show MTGreen fluorescence by cell division with or without mDivi-1 treatment. Trapezoid gate denotes MTGreen-low population. Plots are representative of 3 independent experiments. (c)

Left, unequal levels of aged (pulse-labeled) mitochondria in IRF8-hi and IRF8-int progenitors at day 3 of culture. Right: Quantification of MFI of aged mitochondria in IRF8-hi and IRF8-int progenitors. * $P < 0.05$. Paired T test. (d) Total mitochondrial content (left) and mitochondrial membrane potential (right) in mature cDC1 and cDC2 subsets at day 9 of culture. Histograms are representative of 3 independent experiments. (e) Upper left, gating of subsets with low or high levels of mitoclearance. Upper right, total mitochondrial content in proliferating progenitors stained at day 0. Lower, total mitochondrial content of populations with low (left) or high (right) levels of retained, pulse-labeled mitochondria, gated as in upper left panel. Vertical statistic denotes MFI of Mitotracker staining. (f) Left, frequency of cDC and pDCs amongst CD11c+ DCs following mDivi-1 (upper row) or M1 (lower row) treatment. * $P < 0.05$. n.s. not significant. Student's t -test with Holm-Sidak correction. Right, frequency of IRF8+ and IRF4+ cDC subsets following treatment with mDivi-1 (upper) or M1 (lower). ** $P < 0.01$. *** $P < 0.001$. Paired t -test. (g) Flt3L induces mROS production in proliferating progenitors. Left, elevated MitoSox levels in cells with high versus low levels of old mitochondria. Histograms are representative of 2 independent experiments. Middle, MitoSox levels versus cell division at day 3 of culture within IRF8-int and IRF8-high progenitors. Right, quantification of MitoSox MFI in IRF8-int and IRF8-high cells. **** $P < 0.0001$. Student's t -test. (h) Left, frequency of cDC and pDCs within CD11c+ DCs following treatment with N-Ac. n.s. not significant. Student's t -test with Holm-Sidak correction. Right, frequency of IRF8+ and IRF4+ cDC subsets following treatment with N-Ac. * $P < 0.05$. Paired t -test.

Figure 3.

Nutrient sensor AMPK promotes selective DC differentiation. (a) AMPK signaling in dividing progenitors stimulated by Flt3. Levels of phospho-AMPK, phospho-ACC, and phospho-Raptor in proliferating (black line) and undivided (grey fill) progenitor cells stimulated with Flt3L for 3 days. Plots are representative of 2–3 independent experiments. (b) AMPK- α 1 deficiency alters DC development *in vitro*. Left, development of cDC/pDCs from AMPK- α 1 deficient cells and heterozygote littermate (“WT”) controls. n.s. not significant. Student’s *t*-test with Holm-Sidak correction. Right, differentiation of IRF8⁺ and IRF4⁺ cDC subsets from AMPK- α 1 deficient progenitors with or without mDivi-1

treatment. * $P < 0.05$ ** $P < 0.01$ *** $P < 0.001$. Bar graphs indicate frequency of each subset $\pm$ SEM. Repeated measures one way ANOVA with Tukey's correction. (c) Steady state *in vivo* bone marrow DC progenitor (upper), as well as skin draining lymph node (lower) cDC/pDC and cDC subset frequencies of AMPK- $\alpha 1$ knockout mice and heterozygote littermate (WT) controls. In bone marrow, DC progenitors were gated as lineage (CD3, CD19, B220, NK1.1, Ter119, GR1, CD11b) negative, Flt3R+, CX3CR1+ cells. Macrophage dendritic cell progenitors (MDP) were c-kit+ CD11c-. Common dendritic progenitors (CDP) were c-kit- CD11c-. Pre-DCs were c-kit- CD11c+. In lymph nodes, pDCs were lineage- CD11c+ MHCII-int B220+, and within lineage- CD11c+ MHCII+ B220- cDCs, cDC1 were gated as CD8 α + CD11b-, migratory DCs were CD8 α - CD11b-, and cDC2 were CD8 α - CD11b+. * $P < 0.05$. *** $P < 0.001$. n.s. not significant. Paired *t*-test.

Figure 4.

Asymmetric division of DC progenitors supporting developmental branching. (a) Left, heterogeneity of IRF8 expression by cell division. IRF8-low, IRF8-int, and IRF8-high populations at day 3, IRF8-int and IRF8-high at day 6, and IRF8-low and IRF8-high at day 9. Right, monotonic, late IRF4 expression accompanying DC differentiation at day 3, day 6, and day 9 of culture. Plots are representative of at least 3 experiments. (b) Developmental potential of IRF8-hi and IRF8-int subsets. Left: FACS sorting strategy to isolate CD11c-IRF8-hi and IRF-int subsets at day 4–5. Middle: Differentiation of mature IRF8+ and IRF4+ subsets from each sorted population at day 9 (day 4 after sorting and re-plating). Right:

Frequency of IRF8⁺ and IRF4⁺ cDCs obtained from cultures of IRF8-hi and IRF8-int starting populations. Bars indicate mean $\pm$ SEM. (c) Localization and inheritance of IRF8 in progenitors at day 4–5 of culture with Flt3L. Proliferating cells were FACS sorted for immunofluorescence (IF) microscopy analysis, and representative IF images of IRF8, tubulin, and DNA are shown. Left, IRF8 localization in interphase and metaphase/anaphase cells. Middle, quantification of metaphase IRF8 asymmetry ratio and % asymmetric cells. Right, localization of IRF8 in late cytokinetic cells. Quantification of IRF8 asymmetry ratio and % asymmetric cells. N=10 metaphase cells. N=10 cytokinetic sibling cell pairs. *** $P < 0.001$. n.s. not significant. Fischer's Exact Test. Scale bar 5 μ M. (d) Left, Flt3L induces phosphorylation of FoxO1/3a. Representative histograms and quantification of p-FoxO1/3a in IRF8-high progenitors stimulated with Flt3L overnight or rested without cytokine. * $P < 0.05$. Paired t -test. (e) Nuclear FoxO3a localization in IRF8hi daughter cells. Left panels, representative IF images of IRF8, tubulin, DNA, and transmitted light in conjoined sibling cells. Among IRF8-asymmetric cytokinetic cells (N=14), proportion of IRF8-high (middle panel) or IRF8-lo (right panel) daughters with nuclear (black fill) or cytoplasmic (white fill) FoxO3a localization.