The Transcriptional Repressor Gfi1 Controls STAT3-Dependent Dendritic Cell Development and Function

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Summary

The mechanisms controlling the differentiation of dendritic cells (DCs) remain largely unknown. Using a transcriptional profiling approach, we identified Gfi1 as a novel critical transcription factor in DC differentiation. Gfi1−/− mice showed a global reduction of myeloid and lymphoid DCs in all lymphoid organs whereas epidermal Langerhans cells were enhanced in number. In vivo, Gfi1−/− DCs showed striking phenotypic and functional alterations such as defective maturation and increased cytokine production. In vitro, Gfi1−/− hematopoietic progenitor cells were unable to develop into DCs. Instead, they differentiated into macrophages, suggesting that Gfi1 is a critical modulator of DC versus macrophage development. Analysis of hematopoietic chimeras and retrovirus-reconstituted hematopoietic progenitor cells established a cell autonomous and nonredundant role for Gfi1 in DC development. The developmental defect of Gfi1−/− progenitor cells was associated with decreased STAT3 activation. In conclusion, we have identified Gfi1 as a critical transcription factor that controls DC versus macrophage development and dissociates DC maturation and activation.

Introduction

Dendritic cells (DCs) play a crucial role in the control of the adaptive and innate immune system. Over the last few years, it has become apparent that the DC compartment comprises functionally diverse cell populations with unique properties. Several subpopulations of DCs have been described based on the expression of cell surface markers, functional characteristics and anatomical localization. Murine CD11c+ DCs can be classified into CD8α− and CD8α+ DCs (Anjuere et al., 1999; Vremec et al., 1992). CD8α+ DCs can be further subdivided into CD4−CD8α+ and CD4+CD8α− subsets (Martin et al., 2000). In addition, CD11c−B220+ DCs have been described as counterparts of human plasmacytoid DCs, producing interferon-α in response to viral infections (Asselin-Paturel et al., 2001).

Despite the phenotypic characterization of diverse DC subpopulations, their developmental pathways of differentiation from hematopoietic stem cells (HSCs) remain poorly defined (Ardavin, 2003). HSC differentiation results in the separation of a lymphoid and myeloid pathway, reflected in the generation of committed common lymphoid and common myeloid progenitor cells (Kondo et al., 2003). Early concepts postulated the existence of CD8α+ lymphoid DCs and CD8α− myeloid DCs, originating from common lymphoid progenitors and common myeloid progenitors, respectively (Anjuere et al., 1999; Vremec et al., 1992; Wu et al., 1996). This conceptual dichotomy was challenged by findings from different groups that myeloid progenitor cells can give rise to “lymphoid” DCs and that lymphoid progenitor cells can give rise to “myeloid” DCs (Mantz et al., 2001; Martin et al., 2000; Traver et al., 2000). More recently, committed DC progenitor cells have been identified in bone marrow and peripheral blood (D’Amico and Wu, 2003; del Hoyo et al., 2002). Thus, a more detailed description of specific developmental stages in hematopoietic and DC development has become possible.

The lineage fate of hematopoietic progenitor cells is controlled by an orchestrated expression pattern of transcription factors, yet the precise molecular mechanisms how these factors govern the diversification of dendritic cell progenitor cells remain largely unknown. The analysis of gene-targeted mice has revealed the functional importance of a few critical transcription factors for DC development. Mice lacking RelB (Wu et al., 1998), PU.1 (Guerriero et al., 2000), and Ikaros (Wu et al., 1996) show reduced numbers of CD8α− DCs, whereas mice lacking IRF4 (Suzuki et al., 2004), IRF8 (Schiavoni et al., 2002), and Id2 (Hacker et al., 2003) exhibit a defect in the development of CD8α+ DCs. STAT3 activation has been identified as a crucial checkpoint of Flt3L-regulated DC development. In STAT3−/− hematopoiesis the numbers of common DC precursors as well as their DC progeny are severely reduced (Laouar et al., 2003). Specific transcription factors may therefore govern the development of defined DC subpopulations. However, no clear paradigm of developmental hierarchies has evolved up to date.

We hypothesized that the analysis of transcription factors upregulated during early steps of GM-CSF-dependent DC maturation might elucidate master regulators controlling DC differentiation. We made use of a murine hematopoietic progenitor cell line (FDCP-mix) permissive for in vitro DC differentiation and performed transcriptional profiling analysis. Here, we report that Gfi1, a zinc finger repressor molecule (Zweidler-McKay et al., 1996) previously implicated in hematopoietic stem cell homeostasis (Zeng et al., 2004; Hock et al., 2004), T cell (Schmidt et al., 1998; Karsunky et al., 2004), and B cell (Fredman et al., 2000), regulates the differentiation and maturation of DCs. Gfi1−/− DCs exhibit a defect in the development of CD8α− DCs, whereas Gfi1−/− mice show a global reduction of myeloid and lymphoid DCs in all lymphoid organs whereas epidermal Langerhans cells are enhanced in number.
RNA was therefore isolated at hours 0, 6, 24, and 48 of differentiation during early differentiation steps. Total of green fluorescent protein (Gfi1 GFP/+)( Yücel et al., (upper panel) and lymph nodes (lower panel) of Gfi1 −/− genes was seen at 48h, containing a set of 91 genes, tant implications for the control of normal DC differentiation. We focused pensions from lymphoid organs in Gfi1 −/− and Gfi1 +/+. We generated hematopoietic chimeras by transplanting lineage-depleted hematopoietic progenitor cells in Gfi1 GFP+/+ mice in vivo. We were able to detect GFP expression in CD11c+MHC-classII+ (Figure 1H) and in Flt3+c-kit+lin− precursor DCs (Figure 1I) suggesting that Gfi1 expression is physiologically regulated in DC progenitor cells.

Results

Screening of Gene Expression in Early DC Development and Transcriptional Activity of the Gfi1 Locus

To identify transcription factors that control dendritic cell development, we performed transcriptional profiling analyses of the murine hematopoietic progenitor cell line FDCP-mix that is permissive for DC differentiation in the presence of GM-CSF (Schroeder et al., 2000) (Figure 1A). We reasoned that GM-CSF modulates a defined set of decisive transcription factors governing DC differentiation during early differentiation steps. Total RNA was therefore isolated at hours 0, 6, 24, and 48 of this in vitro differentiation system, hybridized to Affymetrix GeneChips and analyzed by K-means cluster analysis. The most specific cluster for upregulated genes was seen at 48h, containing a set of 91 genes, including 6 transcription factors as presumable master regulators of DC differentiation (Figure 1B). We focused our further studies on Gfi1, a transcriptional repressor with previously known regulatory roles in the hematopoietic system ( Zweidler-McKay et al., 1996; Schmidt et al., 1998; Yücel et al., 2003; Karsunky et al., 2002b; Hock et al., 2004; Zeng et al., 2004; Hock et al., 2004) and could confirm GM-CSF-dependent Gfi1 upregulation by RT-PCR in FDCP-mix and primary Sca-1+lin− hematopoietic progenitor cells (Figure 1C). To assess the physiological expression pattern of Gfi1, we made use of a novel transgenic Gfi1:GFP knockin mutant mouse in which one Gfi1 allele is replaced by the cDNA of green fluorescent protein (Gfi1GFP+/+)( Yücel et al., 2004). We determined the fluorescence intensity of various CD11c+ DC subpopulations in lymphoid organs, comparing Gfi1GFP+/+ and Gfi1+/- mice. GFP expression was detected at various intensities in CD11c+ DCs obtained from spleen and lymph nodes (Figure 1D). We hypothesized that a heterogeneity in GFP expression in vivo might depend on the activation status of DCs. To address this question, we injected Gfi1GFP+ mice i.p. with LPS or stimulating anti-CD40 monoclonal antibodies, sacrificed the mice 24 hr later and prepared single cell suspensions from spleens and lymph nodes. Compared to Gfi1GFP+/+ DCs isolated from mice treated with PBS, GFP expression was enhanced in Gfi1GFP+/+ DCs obtained from mice treated with LPS or anti-CD40 monoclonal antibodies (Figure 1E), suggesting that Gfi1 expression is physiologically increased upon DC activation in vivo. The presence of Gfi1 protein in DCs was also confirmed by Western blot analysis (Figure S2 in the Supplemental Data available with this article online). Next, we analyzed the GFP expression profile in DC development in vitro over time. We purified lineage-depleted hematopoietic progenitor cells from Gfi1+/-, Gfi1GFP+/+ and Gfi1GFP/GFP mice and incubated the cells in the presence of either recombinant GM-CSF or re- combinant Flt3L, two cytokines known to control myeloid and plasmacytoid DC differentiation, respectively. Aliquots of cells were analyzed for GFP expression by flow cytometry every 6–12 hr. Expression of GFP revealed transcriptional activation of the Gfi1 locus during early phases of DC development and a decline at later phases (Figure 1F). However, only in the case of GM-CSF we observed a marked increase in the fluorescence intensity during hours 48–72. In homozygous Gfi1GFP/GFP progenitor cells, the levels of fluorescence intensity were generally higher and followed a defined pattern over time (Figure 1G), consistent with Gfi1 autoinhibition in DC development. To validate these in vitro findings, we next assessed GFP expression in DC progenitor cells in Gfi1GFP/GFP mice in vivo. We were able to detect GFP expression in CD11c+MHC-classII+ (Figure 1H) and in Flt3+c-kit+lin− precursor DCs (Figure 1I) suggesting that Gfi1 expression is physiologically regulated in DC progenitor cells.

Gfi1−/− Mice Show Decreased Numbers of DCs in Lymphoid Organs

We reasoned that the distinct expression profile of Gfi1 at early stages of DC development might have important implications for the control of normal DC differentiation. We enumerated CD11c+ DCs in single cell suspensions from lymphoid organs in Gfi1−/− and Gfi1+/- mice. In Gfi1−/− mice, the absolute numbers of CD11c+ DCs were significantly reduced in lymph nodes (~10-fold), spleen (~2-fold) and thymus (~20-fold) (Table 1). In view of the GM-CSF-specific upregulation of Gfi1 during early DC development, we were interested to find out whether Gfi1 may act as a lineage-specific transcription factor in “myeloid” versus “lymphoid” DC development and repeated the analysis with a specific focus on defined DC subpopulations. As shown in Figure 2A, both conventional CD11c−B220− DCs and plasmacytoid CD11c−B220+ DCs were reduced in spleen (upper panel) and lymph nodes (lower panel) of Gfi1−/− mice. CD4+ and CD8+ DCs were present, albeit in reduced numbers. In spleens, a relative deficiency affected mostly the CD4+ DC compartment (21% versus 40%, Figure 2B upper panel), whereas in peripheral lymph nodes, mostly CD8hi expressing cells appeared reduced in frequency (7% in Gfi1−/− versus 21% in Gfi1+/-, Figure 2B lower panel). Despite the variations in the relative composition of the DC compartment, the absolute numbers of all DC subpopulations were significantly reduced in spleen (Figure 2C, upper panel), lymph nodes (Figure 2C, intermediate panel) and thymus (Figure 2C, lower panel). In striking contrast to DCs in lymphoid organs, we enumerated an increased number of epidermal Langerhans cells (LCs, 40/mm2 in Gfi1+/- versus 92/mm2 in Gfi1−/−) (Figure 2D), suggesting that Gfi1 does not play a critical role in the differentiation of LCs. We attempted to enhance in vivo DC numbers by administration of recombinant Flt3L, a cytokine with known effects on DC progenitors. Interestingly, in contrast to Gfi1+/- mice, Gfi1−/− mice were unresponsive to Flt3L (Figure 2E), further suggesting that nonmyeloid DC development is also perturbed in Gfi1−/− mice.

To assess whether the decreased number of DCs was due to cell-autonomous or rather extrinsic effects, we generated hematopoietic chimeras by transplanting lineage-depleted hematopoietic progenitor cells ob-
Figure 1. Transcriptional Profiling Analysis and Transcriptional Activity of Gfi1 Locus
(A) Cell surface marker analysis of FDCP-mix cells after differentiation into DCs. Open histograms represent expression of indicated markers, and shaded histograms represent isotype controls.
(B) Graphical and numeric representation of expression profile analysis of selected transcription factors upregulated in GM-CSF-treated FDCP-mix cells. Relative expression (normalized to the median) is displayed as color (green = normalized expression level below, black = near to, and red = above the median). Fold Change (hour 48 versus hour 0) is calculated by Affymetrix MAS 5.0, and p values are based on statistical parameters as described for MAS 5.0 software. A comprehensive data set is available in Figure S1 and Array Express database (http://www.ebi.ac.uk/arrayexpress).
(C) Quantification of Gfi1 mRNA expression by RT-PCR in FDCP-mix, and Sca1lin− hematopoietic progenitor cells at hour 0 and 48 hr after GM-CSF treatment. Shown are the mean values of duplicate samples. Data are representative of two independent experiments.
(D) Gfi1 expression in DCs. Gated CD11c+ cells in spleens and lymph nodes from Gfi1+/GFP mice were analyzed for GFP expression (open histogram). Shaded histograms represent the autofluorescence of Gfi1+/+ DCs used as controls. In each experiment, organs from three to five mice were pooled. Data are representative of three independent experiments.
(E) Enhanced transcriptional activity upon DC stimulation in vivo. Gfi1+/GFP mice were i.p. injected with LPS or anti-CD40 monoclonal antibodies and DC fluorescence was measured by flow cytometry 24 hr later (open histograms). Shaded histograms represent the DC fluorescence from PBS-treated Gfi1+/GFP mice.
(F and G) Gfi1 expression in DC development in vitro. Lineage marker-depleted bone marrow cells from Gfi1+/GFP mice (F) and Gfi1GFP/GFP mice (G) were cultured either in the presence of GM-CSF and IL4 or Flt3L, respectively. Cells were harvested every 6–12 hr, and their fluorescence was determined by FACS analysis. Shown is the specific geometric mean fluorescence intensity index calculated as follows: GMFIi = GMFI(Gfi1+/GFP) – GMFI(Gfi1+/+). Results represent the average values of duplicate samples. Data are representative of three independent experiments.
(H and I) Gfi1 expression in precursor DCs. Pooled bone marrow cells from three to five mice were analyzed for GFP expression in CD11c−I-Ab− (H) and Lin−c-kitFlt3+ cells (I). Open histograms represents GFP fluorescence in Gfi1−/− cells, and shaded histogram represents autofluorescence of Gfi1−/− cells. Data are representative of three independent experiments.

Error bars indicate mean ± SEM.
Table 1. Quantification of Dendritic Cells in Lymphoid Organs

<table>
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<tr>
<th>Number of Cells per Organ</th>
<th>Gfi1+/+</th>
<th>Gfi1−/−</th>
<th>Number of DCs per Organ</th>
<th>Gfi1+/+</th>
<th>Gfi1−/−</th>
<th>p Value</th>
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<tr>
<td>Spleen</td>
<td>18 ± 1 x 10⁶</td>
<td>13 ± 4 x 10⁶</td>
<td>2.7 ± 0.5 x 10⁴</td>
<td>1.2 ± 0.4 x 10⁴</td>
<td>&lt;0.0168</td>
<td></td>
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<tr>
<td>Thymus</td>
<td>41 ± 3 x 10⁶</td>
<td>7.0 ± 1 x 10⁶</td>
<td>1.9 ± 0.1 x 10⁴</td>
<td>0.1 ± 0.1 x 10⁴</td>
<td>&lt;0.000007</td>
<td></td>
</tr>
<tr>
<td>PLN</td>
<td>2.7 ± 1.1 x 10⁶</td>
<td>2.7 ± 1.1 x 10⁶</td>
<td>6.7 ± 0.5 x 10³</td>
<td>0.5 ± 0.3 x 10³</td>
<td>&lt;0.000064</td>
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</tr>
<tr>
<td>MLN</td>
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<td>6.0 ± 1 x 10⁵</td>
<td>3.0 ± 0.7 x 10³</td>
<td>0.2 ± 0.03 x 10³</td>
<td>&lt;0.00771</td>
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Gfi1−/− mice (data not shown), confirming previously reported findings (Zeng et al., 2004). HSC give rise to committed progenitors, named CMP (lin−IL7R−c-kit+. Flt3+CD34+) and CLP (lin−IL7R−c-kit+). Both CMP and CLP contain Flt3+ cells that might represent progenitor cells preceding a DC precursor cell. The percentage of FcRγ+CD34+ and CD11c+CD4+ DC frequencies in spleens (upper panel) and lymph nodes (lower panel). Flow cytometric analysis was performed on pooled mononuclear cells from five mice. Shown is a representative experiment of four.

Figure 2. Global Decrease of DCs in Lymphoid Organs of Gfi1−/− Mice
(A) Contour plot indicating relative decrease of conventional (CD11c+B220+) and plasmacytoid (CD11c+B220+) DC frequencies in spleens (upper panel) and lymph nodes (lower panel, PLN). Flow cytometric analysis was performed on pooled mononuclear cells from five mice. Shown is a representative experiment of four.
(B) Contour plots indicating relative decrease of splenic CD11c+DC4+ DC frequencies (upper panels) and relative decrease of CD11c+CD8+ DC frequencies in lymph nodes (lower panels). Cells were gated on the CD11c+ population.
(C) Absolute DC numbers according to classified DC subtypes. Gfi1−/− mice and Gfi1+/+ mice (n = 5) were sacrificed, and single cell suspensions of spleens (upper panel), peripheral lymph nodes (middle panel), and thymi (lower panel) were pooled prior to enumeration. The absolute number was determined based on the relative FACS profile and total number of cells. Shown is the average of two independent experiments.
(D) Quantification of epidermal Langerhans cells. Epidermal sheets from Gfi1−/− and Gfi1+/+ mice were stained with an APC-conjugated monoclonal antibody reacting against I-Ab. Characteristic Langerhans cells were visualized in a fluorescence microscope (upper panels), and their density was determined using calibrated grids (lower panel).
(E) Sustained defects of DC development in Gfi1−/− mice after Flt3L treatment. Gfi1−/− and Gfi1+/+ mice were treated (i.p.) with Flt3L for nine consecutive days. Subsequently, cells were recovered from spleen and analyzed by FACS staining using anti-CD11c monoclonal antibodies. The absolute number of DCs was determined based on the relative FACS profile and total number of cells.
(F) Contour plot documenting DC chimerism upon bone marrow transplantation. Lineage depleted bone marrow cells from Gfi1−/− mice and Gfi1+/+ mice (CD45.2) were transplanted into lethally irradiated congenic recipient mice (CD45.1). Flow cytometric analysis of splenic DCs reveals donor origin in Gfi1−/− transplanted mice and recipient origin in Gfi1−/− transplanted mice after eight weeks of transplantation. Data are representative of two independent experiments.

Error bars indicate mean ± SEM.
CLP cells was found to be significantly reduced in the bone marrow of Gfi1−/− mice (0.03% in Gfi1−/− versus 1% Gfi1+/+ (data not shown), also confirming previously published results (Zeng et al., 2004; Hock et al., 2004). Furthermore, CD11c+ MHC class II− DC precursor cells in bone marrow (0.3% in Gfi1−/− versus 1% in Gfi1+/+, Figure 3A) and peripheral blood (5% in Gfi1−/− versus 15% in Gfi1+/+, Figure 3A) as well as DC progenitor cells characterized by expression of c-kitFlt3lin− (5% in Gfi1−/− versus 18% in Gfi1+/+, Figure 3B) were significantly reduced in relative as well as in absolute numbers (Figure 3C). This suggests that the decreased numbers of DCs in Gfi1−/− mice is primarily due to insufficient production.

Gfi1−/− DCs Show Functional Abnormalities Revealing Distinct Effects in Maturation and Activation Profiles

DC development is impaired in Gfi1−/− mice but not completely abrogated. Since Gfi1 expression is upregulated upon DC activation as documented by enhanced GFP expression in Gfi1GFP/+ mice, we were interested to assess whether the remaining DCs in Gfi1−/− mice were characterized by functional defects. First, we analyzed the expression levels of MHC and costimulatory molecules on DCs as markers of the maturation status in lymphoid organs. In contrast to normal expression levels of MHC class I, CD40, CD80, and CD86, we found a significant reduction in MHC class II expression in all DC subtypes in Gfi1−/− mice compared to Gfi1+/+ controls (Figure 4A). To further assess whether DC maturation could be triggered by a response to microbial components or inflammatory cytokines, we injected Gfi1−/− and Gfi1+/+ mice with anti-CD40 monoclonal antibodies or LPS, respectively. Twenty-four hours after such stimulation, splenic DCs were harvested, stained, and analyzed by flow cytometry. As shown in Figure 4B, Gfi1−/− DCs were refractory to upregulation of the costimulatory molecules CD40, CD80, and CD86 by such treatment. To assess whether these phenotypic abnormalities represent intrinsic rather than nonspecific secondary effects, we have generated DC-chimeric mice by transplanted CD45.2+Gfi1−/− progenitor cells into sublethally irradiated CD45.1+Gfi1−/− recipient mice. Four weeks after transplantation, we discriminated Gfi1−/− and Gfi1+/+ DCs in the spleens of recipient animals based on the expression of CD45.1 and CD45.2. Upon in vivo challenge with anti-CD40 monoclonal antibodies, only Gfi1+/+ DCs showed normal upregulation of CD40 and CD86, while Gfi1−/− DCs were refractory to maturation (Figure S4). These findings suggest that the phenotypic alterations in Gfi1−/− DCs are intrinsic and not due to an altered cytokine milieu in Gfi1−/− mice.

To test whether a decreased level of DC maturation/activation might be associated with decreased production of inflammatory cytokines such as IL12, we purified splenic DCs from Gfi1−/− and Gfi1+/+ mice and stimulated the cells with TNFα or the Toll-like-receptors ligands LPS (TLR2,4) and CpG (TLR9), respectively. Surprisingly, we found that Gfi1−/− DCs showed a higher baseline level of IL12 secretion that could not be further increased upon stimulation, suggesting that maturation (expression of MHC class II and costimulatory molecules) and activation (expression of IL12) of DCs represent the result of at least partially independent and distinct molecular events (Figure 4C). To further characterize the function of Gfi1−/− DCs, we analyzed their capacity to present specific antigens to T cells. We purified splenic DCs from Gfi1−/− and Gfi1+/+ mice and loaded them with Ova peptides SIINFEKL and Ova323–339, two epitopes presented by MHC class I and II, respectively. The cells were incubated with transgenic OT-I and OT-II T cells recognizing Ova peptides in association with MHC class I and class II. As shown in Figure 4D, Gfi1−/− DCs induced a significantly reduced OT-I (upper panel) and OT-II (lower panel) T cell proliferation in vitro. These data suggest that impaired antigen presentation is an intrinsic feature of Gfi1−/− DCs that can not be readily explained by decreased MHC class II expression levels.

Gfi1 Is a Critical Cell-Intrinsic Modulator of DC versus Macrophage Development

To further elucidate the mechanism of decreased DC differentiation and function, we performed DC differen-
Figure 4. Phenotypic and Functional Abnormalities in Gfi1−/− DCs

(A) Decreased MHC class II expression in Gfi1−/− DCs. Mean fluorescence intensity after staining splenic and lymph node CD11c+ cells for MHC class II expression. Shown is the average GMFI of two independent experiments, pooling organs from five mice. Data are representative of six independent experiments.

(B) Histograms indicating that Gfi1−/− DCs are refractory to upregulate costimulatory molecules. Gfi1−/− and Gfi1+/+ mice were injected with LPS or anti-CD40 monoclonal antibodies, respectively. Pooled DCs from three to five mice were analyzed for expression of CD40, CD80, and CD86 (open histograms). Shaded histograms represent expression levels on DCs from PBS injected mice used as negative controls.

(C) Constitutive secretion of IL12 in Gfi1−/− DCs. Splenic DCs from Gfi1−/− and Gfi1+/+ mice were purified and stimulated with TNFα and the TLR ligands CpG and LPS in duplicates. Shown is one representative experiments out of five. Error bars represent standard error in ELISA.

(D) Impaired antigen presentation in Gfi1−/− DCs. Splenic DCs from Gfi1−/− and Gfi1+/+ mice were loaded with peptides and used as antigen-presenting cells to stimulate the proliferation of transgenic OT-I (upper panel) and OT-II cells (lower panel), respectively. T cell proliferation was measured in triplicates by incorporation of 3H-Thymidine. Data are representative of three independent experiments. Error bars indicate mean ± SEM.
in the surviving cells, 18% showed expression of the DC marker CD11c, suggesting a partial reconstitution of DC development in vitro (Figure 5G). More importantly, we also addressed reconstitution of the DC development upon transplantation of retrovirus-transduced HSC in vivo. Sca1lin− HSC from Gfi1−/− mice were transduced either with SF91-Gfi1-ires-GFP or SF91-GFP and transplanted into irradiated recipient mice. Upon reconstitution of the hematopoietic system, the mice were sacrificed and analyzed for DC reconstitution. Compared to mice transplanted with GFP-expressing progenitor cells, we determined that the percentage of CD11c+ cells among GFP-positive cells was significantly higher in mice that had received Gfi1-transduced progenitor cells (4.8%) compared to mice that had received GFP-transduced progenitor cells (0.2%, Figure 5H), suggesting that the retrovirus mediated expression of Gfi1 corrected the developmental defect of Gfi1 deficient DCs.

Deficient DC Development in Gfi1−/− Mice Is Associated with Decreased STAT3 Activation in Progenitor Cells

Previous in vitro data have shown that Gfi1 interacts with PIAS3, a known inhibitor of STAT3 (Rödel et al., 2000). STAT3 has recently emerged as an important mediator of DC differentiation (Laouar et al., 2003). We therefore hypothesized that altered STAT3-signaling may influence the developmental pathway of macrophage versus dendritic cell development. First, we documented a defect in early cytokine-dependent STAT3 signaling using Western blot analysis. Whereas Gfi1+/+ progenitor cells showed evidence of a rapid STAT3 phosphorylation response upon exposure to GM-CSF
Figure 6. Decreased STAT3 Activation in Gfi1−/− Progenitor Cells
(A) Western blot analysis. Lin-depleted bone marrow cells from Gfi1+/+ and Gfi1−/− mice were incubated in the presence of GM-CSF and IL-4. Cell lysates were prepared at indicated time points. STAT3 (top panel) and phosphorylated STAT3-p(Tyr705) (middle panel) was detected using specific monoclonal antibodies. Monoclonal antibodies recognizing actin were used to confirm equal protein loading (bottom panel).

(B) Detection of phosphorylated STAT3-p(Tyr705) in DC progenitors. Lin− BM cells of Gfi1+/+ and Gfi1−/− mice were stimulated in the presence of GM-CSF and IL-4 for 5 min. Upon intracytoplasmic staining with an anti-STAT3-p(Tyr705) monoclonal antibody, c-kit+Flt3+Lin− cells were analyzed by flow cytometry. Open histograms represent cells treated with monoclonal antibodies recognizing phosphorylated STAT3-p, and shaded histograms represent cells treated with the respective isotype control antibodies. Cells cultured in the absence of GM-CSF and IL-4 served as negative controls.

(C) Western blot analysis. Lin-depleted bone marrow cells from Gfi1+/+ and Gfi1−/− mice were incubated in the presence of GM-CSF and IL-4. Cytosolic (upper panel) and nuclear protein fractions (lower panel) were purified at indicated time points. STAT3 was detected using a STAT3-specific monoclonal antibody.

(D and E) Real-time PCR studies showing RNA levels of SOCS3 and PIAS3. Lin− bone marrow cells of Gfi1+/+ and Gfi1−/− mice were stimulated with GM-CSF and IL-4. RNA was extracted at indicated time points and reverse transcribed into cDNA. The expression levels of SOCS3 RNA (D) and PIAS3 RNA (E) were quantified by real-time PCR studies. Shown are the mean values of duplicate samples. Data are representative of three independent experiments.

(F) Western blot analysis showing protein levels of SOCS3 and PIAS3. Lin-depleted bone marrow cells from Gfi1+/+ and Gfi1−/− mice were incubated in the presence of GM-CSF and IL-4. Cell lysates were prepared at indicated time points. SOCS3 (upper panel) and PIAS3 (lower panel) protein was detected using specific polyclonal antibodies. Actin was detected by specific monoclonal antibodies to confirm equal protein loading.

(G) Contour plots indicating reconstitution of DC development upon retroviral gene transfer of constitutively active STAT3 (STAT3c). Bone marrow progenitor cells were transduced with retroviruses encoding GFP (left panel) or STAT3c-GFP (right panel), respectively. Cells were then differentiated in the presence of GM-CSF and IL-4. On day 8 of culture, CD11c expression was determined. Error bars indicate mean ± SEM.

and IL4, STAT3 phosphorylation was severely impaired in Gfi1−/− progenitor cells (Figure 6A). To further confirm cytokine-dependent STAT3 activation in defined Gfi1−/− DC progenitor cells, we analyzed the phosphorylated STAT3 isoform in c-kit+Flt3−lin− cells by FACS. As shown in Figure 6B, STAT3 phosphorylation was absent in Gfi1−/− DC progenitor cells upon stimulation with GM-CSF and IL-4. Next, we isolated lin− hematopoietic progenitor cells from Gfi1+/+ and Gfi1−/− mice and determined nuclear translation of activated STAT3 by Western blot analysis, comparing cytosolic and nuclear protein fractions. Whereas the specific band corresponding to STAT3 protein in the cytosol was comparable in Gfi1−/− and Gfi1+/+ cells (Figure 6C, upper panel), we documented a significant reduction of STAT3 in the nuclear protein fraction at all time points (Figure 6C, lower panel). This is consistent with impaired STAT3 phosphorylation and suggests that the amount of phosphorylated STAT3 that can translocate into the nucleus is limited in the absence of Gfi1.

STAT3 signaling is negatively regulated by two well-defined STAT3 inhibitors, SOCS3 and PIAS3. We reasoned that decreased STAT3 phosphorylation in Gfi1−/− progenitor cells might be due to enhanced negative feedback regulation of STAT3 specific kinases through SOCS3. It has been shown before that the SOCS3 promoter contains Gfi1/Gfi1b binding sites and SOCS3 expression can be repressed by the Gfi1 homologue Gfi1b.
Strikingly, Gfi1-/- progenitor cells showed higher RNA expression levels of SOCS3 (Figure 6D) and also of PIAS3 (Figure 6E) compared to Gfi+/- control. Consistent with these findings, we also documented increased protein expression levels of SOCS3 and PIAS3 in Gfi1-/- deficient progenitor cells (Figure 6F). Finally, to provide direct evidence that defective STAT3 phosphorylation and activation signaling plays a role in the aberrant differentiation of Gfi1-/- dendritic progenitor cells, we performed rescue experiments using a retroviral vector encoding a constitutively active form of STAT3 (STAT3c). Upon expression of this form of STAT3c in Gfi1-/- progenitor cells, we were able to document a partial rescue of DC development as measured by the differentiation of CD11c expressing DCs (Figure 6G).

Discussion

Using a transcriptional screening approach, we have identified Gfi1 as a crucial transcription factor controlling DC development and function. Gfi1 is a transcriptional zinc finger repressor (Zweidler-McKay et al., 1996) originally identified as a target gene for proviral insertions leading to IL2 independent growth in a T cell lymphoma line (Gilks et al., 1993). Gfi1 contains six C2H2 zinc fingers and a transcriptional repressor domain (SNAG). Previous studies suggest that Gfi1 acts as a protooncogene by accelerating T cell proliferation and inhibiting apoptosis and cell cycle arrest (Grimes et al., 1996; Karsunky et al., 2002a; Schmidt et al., 1998). Furthermore, Gfi1 regulates proliferation and differentiation of thymic and peripheral T cells (Doan et al., 2003; Yücel et al., 2003; Zhu et al., 2002). More recently, a more global role of Gfi1 in hematopoiesis has evolved. Analysis of Gfi1-/- mice revealed an unexpected absence of mature neutrophils (Karsunky et al., 2002b; Hock et al., 2003). Moreover, heterozygous mutations in Gfi1 have been identified in rare patients with hereditary neutropenia, suggesting that both loss of function and dominant negative variants of Gfi1 block neutrophil differentiation (Person et al., 2003). Furthermore, Gfi1 is essential to restrict HSC proliferation and to preserve HSC functional integrity (Hock et al., 2004; Zeng et al., 2004).

Here, we provide evidence for yet another role of Gfi1 in controlling the complexity of hematopoietic stem cell differentiation. Gfi1 deficiency leads to a global reduction in the number of DC precursors and their progeny in bone marrow, thymus, spleen and lymph nodes, as well as to incomplete DC maturation and function. Furthermore, Gfi1 appears to be a key regulator of the lineage decision process for precursors to differentiate into DCs or macrophages.

Our original aim was to identify transcription factors controlling specific subsets of DCs, in particular the development of "myeloid" DCs from GM-CSF treated HSCs. Previous reports have implicated a role for PU.1 (Anderson et al., 2000; Guerrero et al., 2000), RelB (Wu et al., 1998), and Ikaros C (Wu et al., 1997) in the differentiation of CD11c-CD8α- "myeloid" DCs. Unexpectedly, we found a global reduction of all DCs in primary and secondary lymphoid organs in Gfi1 deficient mice, irrespective of CD8α expression. Although our data do not exclude the existence of "myeloid" DCs, the analysis of Gfi1-/- mice does not provide any evidence for a distinct myeloid DC lineage. Interestingly, Gfi1 deficiency did not perturb Langerhans cell development. In that respect, Gfi1-deficient mice resemble RelB-/- (Wu et al., 1998) and IkarosC-/- mice (Wu et al., 1997) that are characterized by a deficiency of CD8α- DCs while epidermal LCs appear normal. In contrast, TGFI-/- (Borkowski et al., 1996) and Id2-knockout mice (Hacker et al., 2003) show a complete lack of epidermal LCs, while other DC subpopulations are at least partially preserved, supporting the notion of a distinct LC lineage. Thus, the analysis of Gfi1-/- mice provides additional evidence for a dissociation of DC development in peripheral lymphoid organs and epidermal Langerhans cells.

The decisive factors controlling macrophage versus DC development remain elusive. Various cytokines, such as IL6, TNFα, and interferon-γ induce DC versus macrophage differentiation in vitro (Chomarat et al., 2000; Chomarat et al., 2003; Delneste et al., 2003). Other studies suggest that the Notch ligand Delta-1 inhibits macrophage differentiation while permitting DC differentiation (Ohishi et al., 2001). However, analysis of cytokine knockout mice has not yet revealed any specific factor that dissociates DC and macrophage development. Intracellular mechanisms responsible for DC versus macrophage development are currently not known. In mice doubly deficient for two NFκB subunits (p50-/-RelA-/-), GM-CSF-dependent DC development is severely reduced whereas M-CSF dependent macrophage development appears normal (Ouaaz et al., 2002). In our study, both FLt3L and GM-CSF, two cytokines inducing DC development in wildtype hematopoietic stem cells, drive Gfi1-/- hematopoietic progenitor cells into macrophage differentiation. Thus, Gfi1 is a unique factor governing DC versus macrophage development in vitro. In this respect, Gfi1-deficient hematopoiesis is reminiscent of STAT3-deficient hematopoiesis that is characterized by increased cell autonomous proliferation of cells of the myeloid lineage (Welte et al., 2003) associated with a defect of DC development (Laouar et al., 2003).

The precise biochemical function of Gfi1 is at present under active investigation. Gfi1 shares the same DNA binding and a SNAG (Snail and Gfi1 family of proteins) repression domain with its homologue, Gfi1B (Grimes et al., 1996). Both factors have redundant and unique biological roles in controlling hematopoiesis (Hock et al., 2003; Saleque et al., 2002; Karsunky et al., 2002b; Doan et al., 2003). As a transcription factor, Gfi1 displays activity in the nucleus as a DNA binding transcriptional repressor. In addition, there is evidence for another function of Gfi1 that does not require DNA binding (Rödel et al., 2000). This is mediated by a direct physical interaction of Gfi1 with PIAS3 (protein inhibitor of activated STAT3), a specific inhibitor of STAT3. Using a STAT3-dependent reporter gene assay, earlier studies have suggested that Gfi1 can increase STAT3 signaling by overcoming the inhibitory effects mediated by PIAS3 (Rödel et al., 2000). Consistent with this finding, there would be an impairment of STAT3 signaling in Gfi1-/- cells. Indeed, here we confirm and extend this observation
into a functional model of DC differentiation and provide unequivocal evidence that in the absence of Gfi1, STAT3 phosphorylation and hence very likely its signaling is significantly reduced in early DC progenitor cells. Independent evidence for a critical role of STAT3 in DC development has recently also been proposed by others (Laouar et al., 2003). In these studies STAT3 activation was found to be a critical checkpoint of Flt3L-regulated DC development (Laouar et al., 2003). In the absence of STAT3, the transition of CLP and CMP to trast to these models however, Gfi1−/− DCs reveal a discription of a large number of Gfi1 target gene candidates indicated here where the almost complete lack of inducible STAT3 phosphorylation in the absence of Gfi1 correlates with the abrogation of both Flt3L and GM-CSF-dependent DC differentiation. This suggests that deficient STAT3 signaling is not the only mechanism explaining the phenotype of Gfi1−/− hematopoiesis and that other parallel pathways must exist supporting DC differentiation that are affected by the lack of Gfi1.

In its classical function, Gfi1 acts as a transcriptional repressor and influences the expression of a multitude of downstream effector genes. The recent identification of such a large number of Gfi1 target gene candidates illustrated the complexity of Gfi1-dependent pathways (Duan and Horwitz, 2003). Gfi1 binds to functionally diverse sets of genes in myeloid cells such as JAK3, IL8, c-Myc, and members of the G/EBP family, as was shown by chromatin immunoprecipitation (Duan and Horwitz, 2003). Interestingly, Gfi1 autoregulates its own transcription in primary lymphoid cells (Doan et al., 2004; Yücel et al., 2004). Our experiments indicate a similar phenomenon in DC progenitor cells, since the transcriptional activity of the Gfi1 locus is higher in Gfi1GFP/+ progenitor cells compared to Gfi1GFP−/− cells. Similar GFP expression patterns in response to Flt3L and GM-CSF in the Gfi1GFP/+ progenitor cells may reflect a common regulatory role of Gfi1-mediated suppressive effects in “myeloid” and “lymphoid” DC development.

Previous reports have proposed that DC maturation and DC activation have to be seen as molecularly distinct events, phenotypically characterized by (1) upregulation of surface expression of MHC class II and the costimulatory molecules CD40, CD80, and CD86, and (2) production of inflammatory cytokines (e.g., IL12) (Kaisho and Akira, 2001; Kobayashi et al., 2003; Ouaz et al., 2002). DC maturation is primarily induced upon signaling via Toll-like-receptors (TLR) and CD40. Gfi1−/− DCs show decreased expression of MHC class II and decreased upregulation of costimulatory receptors upon stimulation with LPS and anti-CD40 monoclonal antibodies, a phenotype consistent with impaired DC maturation. TRAF6 has emerged as a point of convergence for both TLR- and CD40-mediated signaling cascades, linking both pathways to NFκB activation. TRAF6−/− deficient DCs show decreased maturation and thus resemble Gfi1−/− DCs (Kobayashi et al., 2003). However, in contrast to Gfi1−/− DCs, TRAF6−/− DCs are characterized by decreased cytokine production (Kobayashi et al., 2003). The analysis of mice deficient for the expression of defined factors regulating NFκB has provided more insights into the complex network regulating DC activation and cytokine production. For example, mice doubly deficient in the NFκB subunits p50 and RelA exhibit a severe reduction in CD8+ and CD8− DCs, while mice doubly deficient in p50 and cRel show impaired CD40L-mediated survival and IL12 production (Ouaz et al., 2002). Furthermore, DCs from mice deficient in the negative NFκB regulator NFκB2 show enhanced expression of activation markers but produce normal levels of cytokines (Speirs et al., 2004). In contrast to these models however, Gfi1−/− DCs reveal a dissociated phenotype characterized by decreased maturation and increased activation. In view of our results documenting decreased STAT3 activation in Gfi1−/− DC progenitors, it is tempting to speculate that the increased cytokine secretion is related to deficient STAT3 activation in DCs. Targeted STAT3 deficiency in the hematopoietic system is associated with chronic enterocolitis, aberrant inflammation and lethality to septic peritonitis (Matsukawa et al., 2003; Takeda et al., 1999). This pathology is associated with increased expression of inflammatory cytokines. No data have been published about maturation of STAT3−/− DCs, but overstimulated innate immunity is associated with enhanced NFκB activity in STAT3−/− hematopoietic cells (Welte et al., 2003). Thus, Gfi1−/− mice offer a model system to further dissect the mechanisms controlling DC maturation and activation.

In conclusion, our results demonstrate a key role for the transcriptional repressor Gfi1 in DC development and function. Our studies provide insights into the complex hierarchical network controlling DC differentiation, maturation and activation, and reveal specific pathways that might ultimately be important for the design of rational DC therapies.

Supplemental Data

Supplemental Data include five additional figures, Experimental Procedures, an additional table, and Supplemental References and can be found with this article online at http://www.immunity.com/cgi/content/full/22/6/717/DC1/.

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