STAT3- and STAT5-dependent pathways competitively regulate the pan-differentiation of CD34^{pos} cells into tumor-competent dendritic cells

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Running title: STAT-regulated DC acquisition of tumor-competence

Scientific category: Immunobiology
Abstract

The clinical outcomes of dendritic cell (DC)-based immunotherapy remain disappointing, with DCs often displaying a tenuous capacity to complete maturation and DC1 polarization in the tumor host. Surprisingly, we observed that the capacity for successful DC1 polarization, including robust IL12p70 production, could be regulated by STAT-dependent events even prior to DC differentiation. Exposure of CD34pos cells to single agent GMCSF induced multilineage, STAT5-dependent differentiation, including DCs which failed to mature in the absence of further exogenous signals. In contrast, Flt3L induced nearly global differentiation of CD34pos cells into spontaneously maturing DCs. IL-6 synergized with Flt3L to produce explosive, STAT3-dependent proliferation of phenotypically undifferentiated cells which nevertheless functioned as committed DC1 precursors. Such precursors not only resisted many tumor-associated immunosuppressants, but also responded to tumor contact or TGFβ with facilitated DC maturation and IL12p70 production, and displayed a superior capacity to reverse tumor-induced T-cell tolerance. GMCSF preempted Flt3L or Flt3L+IL6 licensing by blocking STAT3 activation and promoting STAT5-dependent differentiation. Paradoxically, following overt DC differentiation, STAT5 enhanced whereas STAT3 inhibited DC1 polarization. Therefore, non-overlapping, sequential activation of STAT3 and STAT5, achievable by sequenced exposure to Flt3L+IL6, then GMCSF, selects for multilog expansion, programming and DC1 polarization of tumor-competent DCs from CD34pos cells.

Introduction

Dendritic cells (DCs) are the most potent antigen-presenting cells in the body and are employed in many tumor vaccine immunotherapy trials, rarely with therapeutic impacts.1;2 DC preparations display a wide range of characteristics in vitro and in vivo, and it remains uncertain which individual properties may best promote successful immunotherapy.3-6

A variety of single agents, including CD40 ligand, Toll-like receptor (TLR) agonists, and calcium ionophore, can induce DC phenotypic maturation.3;4;6-9 Such maturation includes pronounced expression of MHC and costimulatory molecules, CD40 and CCR7, and IL-8 secretion, but falls short of the DC’s potential to achieve DC1-polarization, a highly effective state for promoting cell-mediated immunity.4;6;7;9;10 DC1-polarization includes abundant production of IL12p70 heterodimer and IL-23, secretion of the chemokine MIP-1β, and preferential expression of Delta-4 Notch Ligand.4;6;7 Such DC1 products are highly associated with chemoattraction and activation of T1-type CD4+ and CD8+ T-cells.4;6;7 Furthermore, IL12p70 production is critical to sensitize high avidity T-cells which directly recognize and kill tumor targets.4;6;7

Although desirable for anti-tumor immunity, DC1-polarization is more easily signaled by infectious agents than by tumor exposure. Immature DCs employ recognition of pathogen-associated molecular patterns to assess the likelihood of host infection and the appropriateness of DC1-polarization.6;7 Individual TLRs signal DCs primarily through the MyD88 pathway (e.g., TLR7-9) or TRIF pathway (e.g.,TLR3), with TLR4 evidencing pathway duplicity.7 While activation of either pathway can induce elements of phenotypic DC maturation, dual pathway activation, or single pathway activation potentiated by exposure to IFN-γ or CD40 ligation, is required for robust DC1-polarization.6;7
Fresh mobilization of DC1 precursors has the theoretical potential to promote cross-presentation of tumor Ags within the tumor host. Recent studies confirm the capacity of stem cell-mobilizing treatments, notably granulocyte-macrophage colony stimulating factor (GMCSF), granulocyte colony stimulating factor, fms-like tyrosine kinase receptor-3 (Flt3 ligand, Flt3L), or combined Flt3L+GMCSF to mobilize DC precursors, but the potential of such precursors to achieve DC1-polarization is presently unclear. Flt3L+GMCSF mobilization was recently reported to induce abundant infiltration of DCs into mouse tumors, but such DCs also activated regulatory T-cells and promoted tumor tolerance. Immunosuppressive factors including IL-10, TGF-β (transforming growth factor beta), vascular endothelial growth factor (VEGF) and prostaglandin E2 (PGE2) are often produced by tumors, and may impede mobilized DC precursors from attaining optimal maturation and DC1-polarization. However, mobilizing treatments themselves can also influence the later differentiation responses of stem cells. We therefore postulated that particular stem cell proliferative treatments might provide conditioning signals which licensed rather than limited responsiveness to DC1-polarization stimuli.

Stem cell proliferation is inducible via multiple signaling pathways, including receptor-linked tyrosine kinases (Flt3L and stem cell factor (SCF)), gp130 (e.g., IL-6), and the hematopoietin receptor superfamily (e.g., GMCSF). During the systematic testing of such signaling agents we identified that combined exposure to Flt3L plus IL-6 (Flt3L+IL6) not only synergized for stem cell proliferation, but also licensed CD34pos progenitor cells to forego multilineage differentiation in favor of STAT3-dependent, dedicated DC differentiation. Such STAT3-dependent DC differentiation proved highly conducive to DC1-type functional competence during later interactions with tumor. In contrast, exposure of CD34pos cells to GMCSF dominantly promoted an alternative, STAT5-dependent pathway of DC differentiation which was less conducive to tumor interactions.
Materials and Methods

Mice and Tumors All experiments were performed under institutionally approved animal research committee protocols adhering to U.S.D.A guidelines. Female C57BL/6N, C3H/HeJ and BALB/c mice were purchased from Biologic Testing Branch, NCI (Frederick, MD). They were maintained pathogen-free under USDA guidelines and studied at 8-12 weeks or as indicated. MCA-203, 105 and 205 fibrosarcomas and B16 melanoma, syngeneic to C57BL/6N mice; CT26 colonic adenocarcinoma, syngeneic to BALB/c mice; and the 888 mel human melanoma line were maintained as described previously.21,22

After extensive C57BL/6N backcross, STAT5a/b knockout (KO) mice23 with hypomorphic STAT5 expression (N-terminal truncation24) were obtained by breeding heterozygotes to yield viable STAT5ab−/− pups. Mice were genotyped by PCR of tail clip genomic DNA using primer pairs specific for STAT5a and/or STAT5b genes as previously described.25

Following exhaustive C57BL/6N backcross, mice homozygous for a STAT3 allele with loxP sequences flanking critical exons 18-20 (“F allele”) were bred with Tie2-Cre mice (“C” allele”) to generate mice with bone marrow (BM) conditionally knocked out for STAT3 (“STAT3-CFF”).26 Tail clip PCR genotyping distinguished a 490-bp product of wildtype STAT3 from the 520-bp STAT-F product, as well as the presence or absence of a Tie2-Cre 300-bp product.

Reagents rhFlt3L (gift of Amgen, Thousand Oaks, CA). rhIL-2 (gift of Chiron, Emeryville, CA), rmGMCSF (gift of Immunex, Seattle, WA); rmCSF, rmIL-6, rmIL-10, rmVEGF, rmIL-3, rm thrombopoietin, rmIL-4, rmIFN-γ, rhIL-7 and rhIL-15 (Peprotech, Rocky Hill, NJ); rhTGFβ1 (R&D, Minneapolis, MN); PGE2, LPS (E. coli 026:B6), poly I:C and prostaglandin E2 (Sigma, St. Louis, MI); CpG (ODN 1826) and imiquimod (Invivogen, San Diego, CA) were employed. Culture medium (CM) consisted of RPMI-1640 with 10% heat deactivated FCS and conventional additives.21

BM proliferation (Step 1 Culture) Mouse BM suspensions were prepared from femurs and tibias.27 Following RBC lysis by ammonium chloride, 12-15 million BM cells (CD34pos cell frequency 8-12%) were cultured at 0.5 million/ml in 75 cm2 flasks (Corning, New York) at 10% CO2 in CM with the factors to be tested: hFlt3L 25 ng/ml (similar results 25-300 ng/ml); mIL-6 25 ng/ml (similar results 25-100 ng/ml, 100 ng/ml rhIL-6 also effective); mSCF 25 ng/ml (similar results 25-100 ng/ml, rSCF also effective); rmGMCSF 10 ng/ml (similar results 10-25 ng/ml); rmIL-4 10 ng/ml (similar results 10-25 ng/ml). Recombinant thrombopoietin, IL-3 and fibronectin were also assessed, with negligible superimposed proliferative impacts (not shown). BM cells were normally cultured for 6-7 days, harvested, and washed twice in PBS before Step 2 cultures.

In vitro BM differentiation/maturation (Step 2 Culture) Step 2 was initiated (0 hr) in CM, rmGMCSF and optionally rmIL-4 in 24 well cluster plates, 4 million BM cells/well. DC1-polarization stimuli such as CpG (ODN1826, 5 μM) and lipopolysaccharide (LPS, 50 ng/ml) were added between 18-24 hrs, and cells harvested at 40-44 hrs for analyses. When included, rmIL-10, rmVEGF, rhTGFβ1 or PGE2 were added between 0-24 hrs of Step 2 culture. Alternatively, particulate tumor cells, either viable unirradiated, viable irradiated (10,000 cGy), or killed freeze-thawed lysate,28 were added. Viable tumor cells were sometimes labelled with CFSE (Molecular Probes, Eugene, OR) to allow exclusion during FACS analyses.21

FACS Analyses of cultured BM Cells were cultured in anti-CD32 mAb plus normal mouse IgG to block FcR, then stained with fluorescently-conjugated specific or isotype controls mAbs (BD-PharMingen, Mountain View, CA).29 When additionally assessing IL-12 production at the cellular level, the last 16 hrs of Step 2 culture were performed in monensin (Golgistop™, BD-PharMingen). Then, after FcR block and surface molecule staining, cells were treated with CytoPerm/CytoFix™ (PharMingen), then stained with PE-anti-mouse IL12p40 (BD-PharMingen) or PE-isotype control.
Intracellular staining for TLR was performed directly on fixed, permeabilized cells with conjugated mAb (TLR3, TLR4, TLR8, TLR9), or indirectly with unconjugated mAb (TLR7), and appropriate controls from Imgenix (San Diego, CA). Staining for intracellular IRF4 and IRF8 was performed with reagents from Santa Cruz Biotechnology (Santa Cruz, CA) and mouse adsorbed F(ab)² fragments of donkey anti-goat Ab (Research Diagnostics, Inc, Concord, MA), following the method of Tamura et al. ³⁰

To isolate CD34⁺ and CD34⁻ cells from freshly harvested BM, the latter were stained with FITC-rat anti-mouse CD34 (dialyzed to remove sodium azide), then sorted on a FACSARia, yielding >96% pure CD34⁺ and CD34⁻ subpopulations.

In addition to PCR STAT determinations (see above), pSTAT proteins was analyzed on the cellular level by FACS. ³¹ Nuclei of cultured BM cells were permeabilized by sequential exposure to formalin (CytoFix™) and 90% methanol. Cells were then stained with PE- or FITC-conjugated anti-pSTAT3 (pY705), anti-pSTAT5 (pY694) or isotype controls (BD-Biosciences).

**ELISAs** Supematant contents of mIL12p70 heterodimer were quantified with BD-Pharmingen reagents. mIFN-α and mIFN-β were quantified with PBL Biomedical Laboratories kits (New Brunswick, NJ).

**T-cell co-cultures** Prior to T-cell harvest, DCs were prepared under various Step 1 conditions in CM; Step 2 was performed in 1% non heat-deactivated mouse serum (MS) instead of FCS, during which BM cultures were exposed to viable irradiated tumor cells, then CpG+LPS. On the day of DC harvest, T-cells were freshly harvested from tumor-draining lymph nodes (TDLN) of 12 day tumor-bearing mice. The L-selectinlow (tolerized pre-effector) fraction of T-cells was isolated as previously described, ²⁹,³² and cultured in CM-MS with immobilized anti-CD3 ²⁹,³² or variously conditioned DCs. Beginning on day 2 of T-cell culture, some groups also received rhIL-2 (24 I.U./ml), or rhIL-2, rhIL-7 (50 ng/ml) and rhIL-15 (5 ng/ml). T-cells were harvested for assays and adoptive therapy after 12 days of culture.

**T-cell specificity assays (intracellular IFN-γ)** Cultured T-cells were replated in fresh CM-MS at 2 million T-cells/well in 24 well plates. Whole cell irradiated tumor digests (5000 cGy) were added at 2 million cells/well as stimulators. Co-culture proceeded for 18h, with monensin added the final 13 hours. At harvest, individual treatment groups were FcR-blocked, then stained with FITC-anti-CD4 and Cychrome-anti-CD8. Following fixation/permeabilization, cells were additionally stained with PE-anti-mouse IFN-γ or isotype controls, then analyzed.

**Adoptive immunotherapy** 1.5 million viable tumor cells were injected into into healthy syngeneic mice to establish intradermal tumors. ²⁹ 5 or 10 days later, mice received conventional nonmyeloablative whole body irradiation (WBI, 500 cGy), ²⁹ followed by culture-activated T-cells intravenously. Perpendicular bi-dimensional tumor measurements were performed twice weekly. Mice were euthanized when bidimensional product exceeded 225 mm².

**In vivo monitoring of BM cells** Cultured BM cells were labelled with CFSE ²¹ and injected intravenously into syngeneic mice bearing 10 day s.c. MCA-203 or MCA-105 tumors. 48h later, mice were euthanized, tumors harvested and enzymatically digested to produce whole cell digests, with spleen cell suspensions prepared in parallel. ²¹ Preparations from individual mice were analyzed by FACS for CFSE⁺ cell frequencies. Groups were then co-stained with PE-conjugated mAb against DC-associated surface determinants, and FACS gated to analyze the CFSE⁺ subpopulation.

**Statistics:** Survival among treatment groups was compared by Fisher’s exact test. Individual mice were scored for final treatment outcome (lethal tumor vs cure) and treatment groups compared. A two-tailed p value <0.05 was deemed significant. Proliferative synergy was assessed by Wilcoxon
signed rank test for paired data (proliferation following exposure to combined factors vs summed synchronous proliferations of the individual factors). Trafficking accumulation of CFSE-labelled cells and FACS pSTAT quantitations for cultured BM were assessed by Wilcoxon signed rank test for paired data. In all cases, a two-tailed p value <0.05 was deemed significant.
Results:

Later potentials for DC differentiation, DC maturation and DC1-polarization are determined by early CD34\(^{pos}\) cell conditioning

We cultured fresh mouse BM in two steps, a 6-7 day proliferative culture (Step 1) and a 48-72 hour post-proliferative culture (Step 2), followed in some experiments by T cell co-culture (Fig 1a).

Consistent with previous reports, \(^{18-20}\) three treatment pairings (Flt3L+IL6, SCF+IL6, or Flt3L+GMCSF) produced synergistic proliferation during Step 1 culture (Fig 1b). Such expansions represented a selective 35-80 fold numeric expansion of the CD34\(^{pos}\) cell subpopulation, with rapid dropout of initially CD34\(^{neg}\) cells (Fig 1c). CD34\(^{pos}\) cells continued brisk expansion for at least one additional week in culture if replenished with the same treatment pairings (not shown).

Even though Flt3L is myeloproliferative when administered to animals, \(^{5,33}\) Step 1 culture with single agent Flt3L produced poor yields, even when dosing extended up to 300 ng/ml (Fig 1b and not shown). This validated previous reports that single agent Flt3L is poorly proliferogenic \textit{ex vivo} unless BM cultures are seeded at sufficiently high density to confer natural IL-6 supplementation.\(^{34}\)

Prior to initial culture, freshly harvested CD34\(^{pos}\) BM cells rarely displayed DC or other lineage markers (not shown). By the end of Step 1, however, cultures treated with either single agent Flt3L or single agent GMCSF displayed frequent differentiation into immature DCs, based on their dual positivity for CD11c and MHC Class II and low expression of CD40 and B7.2 (Fig 1d, also see Supplemental Fig S1a for full tested panel). Conventional DCs (positive for CD11b\(^{pos}\) but negative for B220\(^{neg}\)) predominated in both instances (Supplemental Fig S1b). Uniformly high MHC Class II expression was a hallmark of Flt3L-induced DC differentiation, whereas GMCSF-induced DCs were heterogeneous and predominantly low in regards to MHC Class II expression (Fig 1d).

Since Flt3L by itself was poorly proliferogenic (Fig 1b), we examined the superimposed impacts of IL-6 or GMCSF, since either agent produced proliferative synergy in conjunction with Flt3L (Fig 1b). BM proliferatively conditioned with Flt3L+IL6 failed to acquire either CD11c or MHC Class II expression, due to a pronounced anti-differentiative effect attributable to IL-6 (Fig 1d). In contrast, BM conditioned with either Flt3L+GMCSF or Flt3L+IL6+GMCSF developed heterogeneous differentiation which closely resembled treatment with GMCSF alone (Fig 1d). Therefore, even though IL-6 could exert a pronounced anti-differentiative effort upon Flt3L cultures, GMCSF could dominantly antagonize the conditioning impacts of both Flt3L and IL6 during Step 1 culture.

Following Step 1 conditioning, Step 2 cultures were exposed to TLR agonists to examine their real-time potentials for DC1-polarization (Fig 1e).\(^{6,7}\) Since both TLR9 and TLR4 were invariably expressed at the end of Step 1 mouse BM cultures (not shown), we standardly employed CpG (ODN 1826) and lipopolysaccharide (LPS) during Step 2 culture to elicit coordinate activation of MyD88 and TRIF pathways.\(^{6,7}\)

Step 1 conditioning with single agent GMCSF rendered a large proportion of cells hyporesponsive or unresponsive to subsequent TLR agonists, as evidenced by limited or absent upregulation of MHC Class II, CD40 and B7.2 expression (Fig 1e/Supplemental Fig S2a). Gr-1 co-expression was a common feature of poorly responsive subpopulation(s) (Supplemental Fig S2b).\(^{35,36}\)

In contrast, Step 1 conditioning with single agent Flt3L licensed consistently high TLR responsiveness, manifested by nearly global phenotypic DC maturation and only scant Gr-1\(^{pos}\) elements during subsequent Step 2 culture (Fig 1e/Supplemental Fig S2b). However, Flt3L’s global licensing impacts were antagonized if GMCSF was also included during Step 1 culture (Fig 1e/Supplemental Fig S2a). Such
inhibition was not observed if initial exposure to GMCSF was deferred until Step 2 of culture (not shown).

In contrast to GMCSF, IL-6 not only produced proliferative synergy in conjunction with Flt3L (Fig 1b/1c), but also promoted Flt3L’s global licensing impacts. Although an undifferentiated state persisted during Step 1 conditioning in Flt3L+IL6 (Fig 1d), nearly the entire expanded CD34pos progenitor cell pool responded to subsequent TLR stimulation with DC differentiation, robust phenotypic maturation, and nearly uniform IL12p70 production (Fig 1e, Fig 2a). IFN-β was co-elicited rather than IFN-α by all tested TLR agonist combinations, indicating that conventional DC differentiation dominated during Step 2 culture (Supplemental Fig S2c). However, as for Flt3L, Flt3L+IL6’s global licensing impacts were abrogated if GMCSF was also included during Step 1 culture (Fig 1e/Supplemental Fig 2a).

The functional consequences of each of these conditioning regimens proved age-independent, with indistinguishable outcomes observed for BM obtained from mice aged 4-80 weeks (not shown). Similar responses were observed in all tested mouse strains.

Prior Flt3L or Flt3L+IL6 conditioning promotes spontaneous DC maturation and consistent TLR/IRF expression

Following lineage commitment, DCs typically remain immature unless they are exposed to exogenous signals such as CD40 ligation, calcium ionophore or TLR agonists. It was observed, however, that BM cells conditioned in single agent Flt3L subsequently displayed spontaneous DC maturation, even when GMCSF was the only exogenous supplement provided during Step 2 culture (Fig 2b/Supplemental Fig S3a). Similar spontaneous DC maturation was also observed following Step 1 proliferative conditioning in Flt3L+IL6, even though the inclusion of IL-6 had delayed the onset of DC differentiation until the onset of Step 2 culture (Fig 1d vs Fig 2b/Supplemental Fig S3a). Spontaneous DC maturation following either Flt3L or Flt3L+IL6 conditioning was even more vigorous when IL-4 was also provided during Step 2 culture (Supplemental Fig S3b). Marked upregulation of the endocytic C-type lectin receptor DEC-205 was a conspicuous component of such spontaneous maturation (Supplemental Fig S3c).

For all conditioning treatments other than Flt3L or Flt3L+IL6, spontaneous Step 2 maturation was either highly attenuated or not observed. Importantly, Flt3L+IL6 was the only factor pairing which resulted both in proliferative synergy during Step 1 culture and in spontaneous DC maturation during Step 2 culture (Fig 2b/Supplemental Fig S3). Even when other conditioning treatments evoked limited elements of DC maturation during Step 1 culture (Fig 1d/Supplemental Fig S1a), such elements spontaneously reverted during Step 2 culture unless exogenous maturational stimuli such as TLR agonists were also provided (Fig 1e vs 2b, full panels in Supplemental Fig S2a vs S3). Flt3L or Flt3L+IL6’s capacity to license spontaneous DC maturation was abrogated, however, if GMCSF was also included during Step 1 conditioning (Fig 2b/Supplemental Fig S3).

We characterized expression elements of the conventional DC’s which dominated Step 2 culture following Flt3L+IL6 conditioning. Expression of TLR3, TLR4, TLR7, TLR8 and TLR9 remained uniformly detectable at all stages of culture, functionally confirmed by these cells’ broad responsiveness to respective TLR agonists (Supplemental Fig S4a/S2c). Interferon-regulatory factors IRF4 and IRF8 were dually expressed both by DC precursors and polarized DC1 (Supplemental Fig S4b), demonstrating a largely homogeneous sequence of differentiation, maturation and polarization following Flt3L+IL6 conditioning.
**Flt3L+IL6 conditioned DC precursors are stimulated rather than inhibited by tumor interactions**

We investigated whether Flt3L+IL6 proliferative conditioning licensed uniform responsiveness to DC1-polarization stimuli even in the presence of putative immunosuppressive factors. We added factors at doses which equaled and exceeded those reported to inhibit the maturation of other DC preparations. IL-10 and VEGF exposure had negligible impacts upon DC1-polarization, whereas TGFβ1 exposure paradoxically enhanced both phenotypic maturation and IL-12 secretion (Fig 2c/2d). Only early PGE2 exposure detectably inhibited TLR agonist-induced phenotypic DC maturation and IL-12 production (Fig 2c/2d). Nonetheless, a large subpopulation of Flt3L+IL6 conditioned BM cells resisted PGE2 inhibition even at the beginning of Step 2 culture (Fig 2c, note the bimodal B7.2 expression), and such resistance became increasingly prevalent within hours of Step 2 culture (Fig 2d and Supplemental Fig S5).

We tested the impact of exposing DCs to voluminous tumor burdens at 16-24 hours of Step 2 culture. To each well containing 4 million preconditioned BM cells, we added either 4 million freeze-thawed (killed) tumor cells; 3 million irradiated (10,000 cGy), trypan-excluding apoptotic tumor bodies; or 2 million unirradiated, actively proliferating tumor cells.

After Step 1 Flt3L+IL6 conditioning, contact with any of these tumor materials accelerated DC phenotypic maturation, mimicking the stimulatory impact of exposure to a single TLR agonist (Fig 3a). Combining such tumor exposure with IFN-γ treatment induced IL12p70 production (Fig 3a), mimicking the impact of combined exposure to IFN-γ plus a single TLR agonist. When other Step 1 conditioning conditions were compared, activating effects of tumor were either highly attenuated or completely absent (Fig 3a).

The capacity of Flt3L+IL6 conditioned DCs to be activated by tumor contact proved strain-independent, occurring even for BALB/c BM-derived DCs despite this strain’s Th2-biasing tendency (not shown). All tested tumor lines stimulated DC maturation after Flt3L+IL6 conditioning, including MCA-205 and MCA-203 sarcomas and B16 melanoma derived from C57BL/6N mice, CT26 colon adenocarcinoma derived from BALB/c mice, and 888mel from a melanoma patient, and was not attributable to endotoxin content (not shown). Both freshly harvested whole cell tumor digests and established tumor lines proved stimulatory, indicating that host stromal cells were unessential, and DC maturation was stimulated whether tumor was syngeneic, allogeneic or xenogeneic (not shown). While fully killed lysate was effective, viable tumor proved more effective. Sequestration of tumor from Flt3L+IL6 conditioned DC precursors by Transwell™ membranes abrogated the activating effects of tumor, underscoring a requirement for direct contact; in contrast, phagocytosis of latex beads did not accelerate maturation (not shown).

**Flt3L+IL6 conditioned DCs promote superior reversal of tumor-induced T cell tolerance**

We examined the capacities of previously conditioned DCs to reverse tolerance in T-cells harvested from mice bearing advanced tumors. L-selectin low T-cells from tumor-draining lymph nodes (TDLN) are naturally sensitized to the relevant tumor but are also tolerized, consequent to the progressive upstream tumor burden. In vitro exposure to anti-CD3, followed by IL-2 stimulation, can reverse tolerance and numerically expand anti-tumor effector T-cells. However, such polyclonal stimulation causes CD8+ T-cells to overgrow CD4+ T-cells, and lacks the element of antigen presentation needed to selectively promote the tumor-specific T-cell subset.

Conditioned DC precursors were transferred to Step 2 culture, exposed to viable irradiated tumor, optionally further activated with CpG+LPS, then co-cultured with tolerized TDLN T-cells from mice bearing the relevant tumor. Flt3L+IL6 conditioned DCs efficiently reversed tolerance and stimulated robust T-cell proliferation, even when exogenous cytokines such as IL-2 were not added to co-culture.
CpG+LPS treatment enhanced but was unessential for such efficacy (not shown). In contrast, DCs prepared after other conditioning treatments typically proved lethal to T-cells, and toxicity could not be prevented by exogenous IL-2, IL-15 and/or IL-7 (Fig 3b/4a). Co-cultures driven by Flt3L+IL6 conditioned DC displayed superior outgrowth of both CD4+ and CD8+ tumor-specific T-cells compared to anti-CD3 treatment (Fig 4b.1/4b.2), and were highly potent when provided as adoptive therapy against early or advanced established tumors (Fig 4c/Supplemental Fig S6).

**Flt3L+IL6 conditioned CD34pos cells achieve spontaneous intratumoral DC maturation in vivo**

Following various Step 1 culture treatments, BM cells were CFSE-labelled and administered to 10 day tumor-bearing mice. Cells injected immediately after Step 1 conditioning with Flt3L+GMCSF or Flt3L+GMCSF+IL6 displayed negligible trafficking into either tumor or spleen. In contrast, Flt3L+IL6 conditioned BM cells infiltrated both established tumors and spleen, achieving essentially uniform DC differentiation at either location (Fig 5a/5b). Moreover, accelerated DC maturation was observed following entry into tumor compared to spleen (Fig 5b), consistent with observed stimulatory impacts of tumor contact in vitro during Step 2 culture (Fig 3a).

**Flt3L- vs GMCSF-conditioned DC programming reflects competing STAT3- vs STAT5-dependent events**

Knockout studies have demonstrated that Flt3 ligation transitions CD34pos common precursors into committed DC precursors via a STAT3-dependent process, whereas GMCSF promotes STAT3-independent myeloid differentiation. IL-6 signaling is known to induce gp130-mediated STAT3 activation in CD34pos cells, whereas GMCSF induces more complex STAT modulations, including STAT5 activation, at several stages of myeloid differentiation. Because Step 1 GMCSF consistently abrogated the unique conditioning impacts of Flt3L or Flt3L+IL6, we examined whether STAT modulations played a pivotal role in GMCSF’s apparently dominant regulation.

Step 1 IL-6 in the absence of GMCSF produced sustained activation of STAT3 but not STAT5, as evidenced by intranuclear staining for pSTAT3 (pY705) and pSTAT5 (pY694) (Fig 6a, “End Step 1” and Supplemental Fig S7). In contrast, inclusion of GMCSF as a component of any Step 1 regimen depressed STAT3 activation and produced biphasic upregulation of pSTAT5 (Fig 6a, “End Step 1” and Supplemental Fig S7). These distinctive pSTAT expression patterns modulated again during Step 2 culture, however, when cultures previously conditioned in Flt3L+IL6 now displayed the greatest capacity for STAT5 activation (Fig 6a, “End Step 2”).

We next examined how STAT knockout BM preparations responded to Flt3L+IL6 conditioning. Consistent with STAT3’s putative obligate role in Flt3L-induced DC differentiation, we observed that STAT3KO BM could not survive Flt3L+IL6 in vitro conditioning (not shown). In contrast, STAT5KO BM responded to Flt3L+IL6 conditioning with intact robust proliferation (not shown) and nearly uniform DC differentiation during subsequent Step 2 culture (Fig 6b, row 2). Nonetheless, compared to wildtype littermates, Flt3L+IL6 conditioned STAT5KO DCs displayed submaximal DC maturation and IL12p70 production (Fig 6b, row 2 vs 1, and Fig 6d left). Therefore, the proliferative and differentiative impacts of Flt3L+IL6 conditioning were absolutely STAT3-dependent, whereas subsequent phenotypic maturation and DC1-polarization were at least partially STAT5-dependent.

We also examined how STATKO BM preparations responded to GMCSF-containing Step 1 regimens (GMCSF alone, Flt3L+GMCSF, or Flt3L+GMCSF+IL6). All of these GMCSF-based conditioning regimens were strikingly ineffective for generating DCs from STAT5KO BM, instead yielding predominantly Gr-1pos, MHC Class IIneg cells which displayed morphologic features of mature neutrophils (e.g., Fig 6b, row 4 and not shown). In contrast, STAT3KO BM displayed normal proliferative kinetics (not shown) and typical heterogeneous differentiation, including DC differentiation, in response to all GMCSF-based regimens (Fig 6c, row 4). Following GMCSF-based conditioning, however, STAT3KO...
DCs displayed an abnormally heightened maturational and IL12p70 response to TLR stimulation (Fig 6c, row 4 vs 3 and Fig 6d right). Therefore, all tested GMCSF-containing regimens required intact STAT5 for DC differentiation, and later phenotypic maturation and DC1-polarization displayed the capacity for negative regulation through STAT3.
Discussion

Certain timely stimuli can condition DCs to adhere to an extended period of programming. For example, we recently reported that exposure of monocyte-derived human DCs to IFNγ+LPS not only stimulated an initial burst of IL12p70 secretion, but also licensed a second burst of IL12p70 secretion even days later if CD40 ligation was experienced.6

We report here that durable DC programming can also be secured at remarkably early stages of hematopoiesis, even prior to discernible phenotypic DC lineage commitment. Exposure of fresh mouse BM to Flt3L+IL6 triggered multilog expansion of CD34pos progenitor cells, and committed nearly all cells to subsequent DC differentiation. Such programming included subsequent spontaneous upregulation of MHC/costimulatory molecules, as well as nearly uniform responsiveness to DC1-polarization stimuli. Moreover, proliferative conditioning with Flt3L+IL6 conferred progressive resistance to many tumor-associated immunosuppressive factors, as well as the capacity to respond to either tumor contact or to TGFβ with facilitated DC1-polarization.

Flt3L+IL6 treatment produced identical licensing whether performed upon unfractionated BM or upon sorted CD34pos cells, indicating that more primitive (CD34neg/Sca-1pos) hematopoietic stem cells were not essential conditioning targets. Pronounced biasing towards ultimate DC differentiation was observed whether Step 1 conditioning was performed in Flt3L+IL6 or Flt3L alone. However, in the absence of co-conditioning by IL-6, Flt3L-induced DC precursors remained scant in number and displayed early maturation, whereas the inclusion of IL-6 produced an immense proliferative pool of CD11cneg/MHC Class IIneg cells which were nonetheless already committed to subsequent DC differentiation. Such still phenotypically undifferentiated cells displayed the capacity both for wide distribution following i.v. injection and for spontaneous DC differentiation/maturation after entry into tumor.

IL-6 and its receptor transducing component gp130 have long been recognized to synergize for stem cell proliferation with receptor tyrosine kinase-activating stimuli, including both c-kit ligand (SCF) and Flt3-L,18-20 but only combined Flt3L+IL6 also preconditioned for nearly global differentiation of expanded CD34pos cells into spontaneously maturing DCs. Paradoxically, although GMCSF also synergized with Flt3L to induce Step 1 stem cell proliferation, GMCSF antagonized every DC-licensing effect attributable to Flt3L.

Our evidence supports the existence of at least two discrete pathways for conventional DC differentiation with strikingly divergent functional outcomes, a Flt3L-promoted STAT3-dependent pathway and a GMCSF-promoted STAT5-dependent pathway (schematized in Fig 7). The Flt3L-promoted pathway was highly potentiated by IL-6, most likely due to the latter’s capacity to confer sustained STAT3 activation during extended CD34pos cell proliferation, and biased for differentiation into DC precursors which were uniformly facilitated for DC1-polarization, spontaneous DC maturation, and activation by tumor contact. In contrast, the GMCSF promoted, STAT5-dependent pathway gave rise to diverse myeloid elements, including DCs which failed to mature in the absence of further downstream driving signals such as TLR agonists.

We hypothesize that STAT5-dependent DC differentiation constitutes the normally dominant pathway, based on several observations: (1) the inclusion of GMCSF during CD34pos progenitor cell proliferation blocked STAT3 activation and promoted STAT5-dependent myeloid differentiation regardless of whether Flt3L or Flt3L+IL-6 were also present; (2) recent studies indicate that STAT3-dependent DC differentiation is not normally detectable in adult mice.42 Nonetheless, we have observed that BM even from much older mice remains highly responsive to exogenous Flt3L or Flt3L+IL6 conditioning (not shown). STAT3-dependent preemptions of normal hematopoiesis in favor of dedicated DC production may therefore reflect a reserved host response, perhaps to life threatening infection of the BM itself. Consistent with this possibility, Flt3L and IL-6 are strongly induced by myelosuppression and BM inflammation, respectively.43,44
Despite the competing roles played by STAT3 and STAT5 in DC differentiation, it is also apparent that their respective impacts change before and after DC differentiation occurs. Pronounced sequential STAT3/STAT5 activation (i.e., Flt3L+IL6 conditioned DCs in Fig 6a) corresponded to the greatest observed attainment of DC tumor-competence, and neither STAT3 nor STAT5 knockout BM responded optimally to Flt3L+IL6 conditioning. The preemptive impacts of GM-CSF during Step 1 culture appear to be mediated through early combined STAT3 inhibition and STAT5 activation (Fig 6). Paradoxically, however, appropriately delayed exposure to GMCSF may promote maximal DC1-polarization through the identical STAT modulations (Fig 7).

The mechanism(s) causing Flt3L+IL6 preconditioned DCs to respond to tumor as a maturational signal remains to be elucidated. A plausible mechanism involves DC activation via enhanced expression of lectin receptors such as asialoglycoprotein receptor and DEC-205 (Supplemental Fig S3c), since identical or similar carbohydrate receptors are employed by tumoricidal macrophages to bind and kill tumor cells in an MHC-unrestricted and antigen-unrestricted manner. Many tumor cells bind lectin more avidly than non-transformed cells, due to a chronically high density of exposed carbohydrates and a diminished presence of differentiation elements which normally mask such carbohydrates. Similarly, we have observed that exposure to albumin crosslinked with mannose or N-acetyl-glucosamine accelerates the maturation of Flt3L+IL6 conditioned BM cells (not shown).

We are investigating why most proliferative conditioning treatments caused DC preparations to be highly toxic to T-cell cultures. Such lethality was unlikely attributable to activation of regulatory T-cells or indoleamine dioxygenase-expressing pDC, since toxicity was not remedied by adding exogenous IL-2 to the T-cell co-cultures. There was, however, a correlation between the Gr-1pos cell-inducing tendency of individual conditioning treatments and observed lethality. It is therefore possible that the Gr-1pos subpopulation(s) mediate the untoward effects of many of the conditioning treatments. Consistent with this possibility, heightened induction of Gr1pos myeloid suppressor cells appears to be the basis of immune disruption by high GMCSF-producing vaccine formulations. In contrast to GMCSF-conditioned cultures, Flt3L+IL6 conditioned DC cultures were exceptional for their low Gr1pos content and consistent absence of toxicity, even when added to T-cells in high proportions.

The above experiments, as well as preliminary studies with human CD34pos progenitor cells (not shown), demonstrate that Flt3L+IL6 may provide an effective means to proliferate, condition and mobilize highly therapeutic DC precursors for tumor therapy. It has long been appreciated that IL-6 has extremely potent therapeutic properties against established mouse tumors, even when administered as a single agent. We postulate that the major mechanistic role and benefit of IL-6 therapy will be in tandem with Flt3L to proliferate and condition tumor-competent DC precursor populations in cancer patients.
Acknowledgements

This work is supported by NIH grants R01-CA089511, R01-CA103946 and RO1-CA129815. There are no competing financial interests.

P.A.C. wrote the paper, designed the experiments, performed experiments and analyzed data. G.K.K., S.S., B.J.C., J.F.T., K.D.B., X.-Y.-F and C.S.C. participated in experimental design and data analyses. Z.W., W.-J.Z., M.A., C.A.D., H.N., C.C.P. and T.D.J. participated in experimental performance and data analyses.

We wish to thank Dr. Walter Storkus for his extremely helpful suggestions.
References

1. Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. Nat.Med. 2004;10:909-915.

2. Banchereau J, Palucka AK, Dhodapkar M et al. Immune and clinical responses in patients with metastatic melanoma to CD34(+) progenitor-derived dendritic cell vaccine. Cancer Res. 2001;61:6451-6458.

3. Bonifaz L, Bonnyay D, Mahnke K et al. Efficient targeting of protein antigen to the dendritic cell receptor DEC-205 in the steady state leads to antigen presentation on major histocompatibility complex class I products and peripheral CD8+ T cell tolerance. J.Exp.Med. 2002;196:1627-1638.

4. Xu S, Koski GK, Faries M et al. Rapid high efficiency sensitization of CD8+ T cells to tumor antigens by dendritic cells leads to enhanced functional avidity and direct tumor recognition through an IL-12 dependent mechanism. J.Immunol. 2003;171:2251-2261.

5. Berhanu A, Huang J, Alber SM, Watkins SC, Storkus WJ. Combinational FLT3 ligand and granulocyte macrophage colony-stimulating factor treatment promotes enhanced tumor infiltration by dendritic cells and antitumor CD8(+) T-cell cross-priming but is ineffective as a therapy. Cancer Res. 2006;66:4895-4903.

6. Czerniecki BJ, Koski GK, Koldovsky U et al. Targeting HER2-neu in early breast cancer development using dendritic cells with staged Interleukin-12 burst secretion. Cancer Res. 2007;67:1-11.

7. Napolitani G, Rinaldi A, Bertoni F, Sallusto F, Lanzavecchia A. Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. Nat.Immunol. 2005;6:769-776.

8. Czerniecki BJ, Carter C, Rivoltini L et al. Calcium ionophore treated peripheral blood monocytes and dendritic cells rapidly display characteristics of activated dendritic cells. J.Immunol. 1997;159:3823-3837.

9. Mailliard RB, Wankowicz-Kalinska A, Cai Q et al. alpha-type-1 polarized dendritic cells: a novel immunization tool with optimized CTL-inducing activity. Cancer Res. 2004;64:5934-5937.

10. Gajewski TF, Renauld JC, Van Pel A, Boon T. Costimulation with B7-1, IL-6, and IL-12 is sufficient for primary generation of murine antitumor cytolytic T lymphocytes in vitro. J.Immunol. 1995;154:5637-5648.

11. Avigan D, Wu Z, Gong J et al. Selective in vivo mobilization with granulocyte macrophage colony-stimulating factor (GM-CSF)/granulocyte-CSF as compared to G-CSF alone of dendritic cell progenitors from peripheral blood progenitor cells in patients with advanced breast cancer undergoing autologous transplantation. Clin.Cancer Res. 1999;5:2735-2741.

12. Pulendran B, Banchereau J, Burkeholder S et al. Flt3-ligand and granulocyte colony-stimulating factor mobilize distinct human dendritic cell subsets in vivo. J.Immunol. 2000;165:566-572.

13. Parajuli P, Mosley RL, Pisarev V et al. Flt3 ligand and granulocyte-macrophage colony-stimulating factor preferentially expand and stimulate different dendritic and T-cell subsets. Exp.Hematol. 2001;29:1185-1193.
14. Kobie JJ, Wu RS, Kurt RA et al. Transforming growth factor beta inhibits the antigen-presenting functions and antitumor activity of dendritic cell vaccines. Cancer Res. 2003;63:1860-1864.

15. Gabrilovich D, Ishida T, Oyama T et al. Vascular endothelial growth factor inhibits the development of dendritic cells and dramatically affects the differentiation of multiple hematopoietic lineages in vivo. Blood 1998;92:4150-4166.

16. Yang AS, Lattime EC. Tumor-induced interleukin 10 suppresses the ability of splenic dendritic cells to stimulate CD4 and CD8 T-cell responses. Cancer Res. 2003;63:2150-2157.

17. Kalinski P, Schuitemaker JH, Hilkens CM, Kapsenberg ML. Prostaglandin E2 induces the final maturation of IL-12-deficient CD1a+CD83+ dendritic cells: the levels of IL-12 are determined during the final dendritic cell maturation and are resistant to further modulation. J. Immunol. 1998;161:2804-2809.

18. Ebihara Y, Tsuji K, Lyman SD et al. Synergistic action of Flt3 and gp130 signalings in human hematopoiesis. Blood 1997;90:4363-4368.

19. Sui X, Tsuji K, Tanaka R et al. gp130 and c-Kit signalings synergize for ex vivo expansion of human primitive hematopoietic progenitor cells. Proc. Natl. Acad. Sci. U.S.A 1995;92:2859-2863.

20. Brasel K, McKenna HJ, Charrier K et al. Flt3 ligand synergizes with granulocyte-macrophage colony-stimulating factor or granulocyte colony-stimulating factor to mobilize hematopoietic progenitor cells into the peripheral blood of mice. Blood 1997;90:3781-3788.

21. Peng L, Kjaergaard J, Plautz GE et al. Tumor-induced Lselectin high suppressor T cells mediate potent effector T cell blockade and cause failure of otherwise curative adoptive immunotherapy. J. Immunol. 2002;169:4811-4821.

22. Parkhurst MR, DePan C, Riley JP, Rosenberg SA, Shu S. Hybrids of dendritic cells and tumor cells generated by electrofusion simultaneously present immunodominant epitopes from multiple human tumor-associated antigens in the context of MHC class I and class II molecules. J. Immunol. 2003;170:5317-5325.

23. Teglund S, McKay C, Schuetz E et al. Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses. Cell 1998;93:841-850.

24. Cui Y, Riedlinger G, Miyoshi K et al. Inactivation of Stat5 in mouse mammary epithelium during pregnancy reveals distinct functions in cell proliferation, survival, and differentiation. Mol. Cell Biol. 2004;24:8037-8047.

25. Bunting KD, Bradley HL, Hawley TS et al. Reduced lymphomyeloid repopulating activity from adult bone marrow and fetal liver of mice lacking expression of STAT5. Blood 2002;99:479-487.

26. Welte T, Zhang SS, Wang T et al. STAT3 deletion during hematopoiesis causes Crohn's disease-like pathogenesis and lethality: a critical role of STAT3 in innate immunity. Proc. Natl. Acad. Sci. U.S.A 2003;100:1879-1884.

27. Kuriyama H, Shimizu K, Lee W et al. Therapeutic vaccine generated by electrofusion of dendritic cells and tumour cells. Dev. Biol. (Basel) 2004;116:169-178.

28. Cohen PA, Cohen PJ, Rosenberg SA, Mule JJ. CD4+ T-cells from mice immunized to syngeneic sarcomas recognize distinct, non-shared tumor antigens. Cancer Res. 1994;54:1055-1058.
29. Peng L, Kjaergaard J, Weng DE et al. Helper-independent CD8+/CD62L low T Cells with broad anti-tumor efficacy are naturally sensitized during tumor progression. J.Immunol. 2000;165:5738-5749.

30. Tamura T, Tailor P, Yamaoka K et al. IFN regulatory factor-4 and -8 govern dendritic cell subset development and their functional diversity. J.Immunol. 2005;174:2573-2581.

31. Onai N, Obata-Onai A, Tussiwand R, Lanzavecchia A, Manz MG. Activation of the Flt3 signal transduction cascade rescues and enhances type I interferon-producing and dendritic cell development. J.Exp.Med. 2006;203:227-238.

32. Wang L-X, Huang W-X, Graor H et al. Adoptive immunotherapy of cancer with polyclonal, 10^8-fold hyperexpanded, CD4+ and CD8+ T cells. J.Translational Med. 2004;2:41.

33. Maraskovsky E, Brasel K, Teepe M et al. Dramatic increase in the numbers of functionally mature dendritic cells in Flt3 ligand-treated mice: multiple dendritic cell subpopulations identified. J.Exp.Med. 1996;184:1953-1962.

34. Brasel K, De Smedt T, Smith JL, Maliszewski CR. Generation of murine dendritic cells from flt3-ligand-supplemented bone marrow cultures. Blood 2000;96:3029-3039.

35. Kusmartsev S, Nefedova Y, Yoder D, Gabrilovich DI. Antigen-specific inhibition of CD8+ T cell response by immature myeloid cells in cancer is mediated by reactive oxygen species. J.Immunol. 2004;172:989-999.

36. Serafini P, Carbley R, Noonan KA et al. High-dose granulocyte-macrophage colony-stimulating factor-producing vaccines impair the immune response through the recruitment of myeloid suppressor cells. Cancer Res. 2004;64:6337-6343.

37. Iborra S, Abanades DR, Parody N et al. The immunodominant T helper 2 (Th2) response elicited in BALB/c mice by the Leishmania LiP2a and LiP2b acidic ribosomal proteins cannot be reverted by strong Th1 inducers. Clin.Exp.Immunol. 2007;150:375-385.

38. Laouar Y, Welte T, Fu XY, Flavell RA. STAT3 is required for Flt3L-dependent dendritic cell differentiation. Immunity. 2003;19:903-912.

39. Majka M, Ratajczak J, Villaire G et al. Thrombopoietin, but not cytokines binding to gp130 protein-coupled receptors, activates MAPKp42/44, AKT, and STAT proteins in normal human CD34+ cells, megakaryocytes, and platelets. Exp.Hematol. 2002;30:751-760.

40. Lehtonen A, Matikainen S, Miettinen M, Julkunen I. Granulocyte-macrophage colony-stimulating factor (GM-CSF)-induced STAT5 activation and target-gene expression during human monocyte/macrophage differentiation. J.Leukoc.Biol. 2002;71:511-519.

41. Giron-Michel J, Fogli M, Gaggero A et al. Detection of a functional hybrid receptor gamma/c/CSFRbeta in human hematopoietic CD34+ cells. J.Exp.Med. 2003;197:763-775.

42. Kortylewski M, Kujawski M, Wang T et al. Inhibiting Stat3 signaling in the hematopoietic system elicits multicomponent antitumor immunity. Nat.Med. 2005;11:1314-1321.

43. Chklovskia E, Jansen W, Nissen C et al. Mechanism of flt3 ligand expression in bone marrow failure: translocation from intracellular stores to the surface of T lymphocytes after chemotherapy-induced suppression of hematopoiesis. Blood 1999;93:2595-2604.
44. Gaugler MH, Squiban C, Claraz M et al. Characterization of the response of human bone marrow endothelial cells to in vitro irradiation. Br.J.Haematol. 1998;103:980-989.

45. Valladeau J, Duvert-Frances V, Pin JJ et al. Immature human dendritic cells express asialoglycoprotein receptor isoforms for efficient receptor-mediated endocytosis. J.Immunol. 2001;167:5767-5774.

46. Cohen PA, Peng L, Plautz GE et al. CD4+ T Cells in Adoptive Immunotherapy and the Indirect Mechanism of Tumor Rejection. Crit.Rev.Immunol. 2000;20:17-56.

47. Oda S, Sato M, Toyoshima S, Osawa T. Binding of activated macrophages to tumor cells through a macrophage lectin and its role in macrophage tumoricidal activity. J.Biochem.(Tokyo) 1989;105:1040-1043.

48. Powell DJ, Jr., Parker LL, Rosenberg SA. Large-scale depletion of CD25+ regulatory T cells from patient leukapheresis samples. J.Immunother. 2005;28:403-411.

49. Munn DH, Sharma MD, Hou D et al. Expression of indoleamine 2,3-dioxygenase by plasmacytoid dendritic cells in tumor-draining lymph nodes. J.Clin.Invest 2004;114:280-290.

50. Mule JJ, Custer MC, Travis WD, Rosenberg SA. Cellular mechanisms of the antitumor activity of recombinant IL-6 in mice. J.Immunol. 1992;148:2622-2629.
**Figure 1: Legend on next page**
Figure 1: Numeric expansion and differentiation following exposure of bone marrow (BM) to various factors. (a) Schematic of test culture system. (b) BM freshly harvested from C57BL/6 mice was cultured for 6 days (Step 1 culture) in the specified factors (dosing in Methods), then counted. Each bar represents averaged 3–11 determinations ± s.d., each performed in synchronous comparison to at least five other groups. Asterixed bars indicate treatment combinations which displayed significant proliferative synergy (see Methods). (c) Fresh uncultured mouse BM cell suspensions were mAb-stained and FACS sorted to separate CD34^pos and CD34^neg subpopulations. CD34^pos cells initially represented 10.8±7% of the total BM cells. The following groups were then subjected to 6 day Step 1 culture either in Flt3L+SCF+IL6 (striped bars) or in Flt3L+IL6 (solid bars): “Unfractionated BM” (12–15 million per flask); “FACS sorted CD34^neg cells” (12–15 million per flask); “FACS sorted CD34^pos cells” (2–3 million per flask); or “FACS sorted, unirradiated CD34^pos cells plus irradiated (3,000 cGy) CD34^neg cells.” Labels above bars indicate fold numeric expansion during the 6 day culture. All numeric expansion observed was attributable to proliferation of the CD34^pos subpopulation, with no CD34^neg feeder layer requirement. (“N.T.”=condition not tested). (d) Surface expression profiles at end of 6 day Step 1 culture in various conditioning treatments. Individual treatments are listed in far left column. 10 different treatments were synchronously compared (all groups shown in Supplemental Fig 1a). Number within each histogram plot indicates the “mean fluorescence specificity index” for the molecule tested, defined as the geometric mean fluorescent intensity of all cells after staining with the specified mAb (filled histogram), divided by the geometric background staining intensity for isotype control mAb (unfilled histogram). Results shown are representative of three comprehensive comparisons. (e) Following the Step 1 conditioning treatments listed in far left column, each group was replated for Step 2 in fresh medium with GMCSF+IL4 for 24 hours, followed by overnight exposure to paired TLR agonists (CpG ODN 1826 and LPS). FACS analyses were then performed on day 2 of this Step 2 culture. 10 different conditioning treatments were compared (selected groups shown in Fig 1e; all groups shown in Supplemental Figure 2a online). Far right column shows gross numeric expansion during initial Step 1 culture. Number within each histogram is the calculated “mean fluorescence specificity index” (see Fig 1d). Representative of three comprehensive comparison experiments.
Figure 2: Stem cells conditioned in Flt3L+IL6 are licensed for DC1-polarization, spontaneous maturation, and resistance to tumor-associated immunosuppressive factors. (a) Production of IL-12 by Flt3L+IL6 conditioned BM cells following Step 2 exposure to CpG+LPS. Culture performed as in Fig 1d, comparing outcomes with or without CpG+LPS treatment. FACS dot plots show results of intracellular cytokine assays for IL12p40 production. %’s shown in right upper quadrants are % of total BM cells specifically staining dually positive for MHC Class II and intracellular IL-12 at the end of culture. Numbers in far right column show ELISA content of IL12p70 heterodimer from culture supernatants run in parallel without monensin (see Methods). Representative of eight experiments. (b) Step 2 culture as in Fig 1e, except following Step 1 conditioning each group was replated in fresh medium for 72 hours solely with rGMCSF (no IL4 or TLR agonists), after which FACS analyses were performed (selected groups shown in Fig 2b; all groups shown in Supplemental Fig 3a online). Number within each histogram is the calculated “mean fluorescence specificity index” at 72h. Results shown are representative of three comprehensive comparisons. A similar but even more pronounced pattern of spontaneous maturation was observed during 48–72 hour Step 2 culture in GMCSF+IL4 (Supplemental Fig 3b online). (c) Step 2 cultures performed as in Fig 1e except that tumor-associated immunosuppressive factors were also added at 0 hr as listed in far left column. This is representative of three experiments. (d) Same as in (a), except listed immunosuppressive factors were added at 0 hr, 1 hr, 2 hr, or 4 hr of Step 2 culture, with supernatants analyzed by ELISA in triplicate for IL12p70 content at 44 hr of Step 2 culture. Error bars indicate s.d. This is representative of three experiments.
Figure 3: Impact of tumor on conditioned BM cells, and impact of tumor-pulsed DCs upon subsequent T cell co-cultures. (a) Following Step 1 conditioning treatments listed in far left column, each group was replated at 4 million cells/well in fresh medium with only GM-CSF added at the beginning of Step 2 culture. 24 hr later, individual wells were also exposed to 3 million viable irradiated MCA-205 tumor cells. FACS analyses of cells were performed at 44 hr (20 hr after addition of tumor). Histograms display expression supernatant content of IL12p70 heterodimer 20 hr after tumor exposure when rmIFN-γ was also added to culture. Supertant IL12p70 content was below detection (<31 pg/24h) following exposure to either rmIFN-γ or to tumor alone (not shown). This is representative of three full comparison experiments. (b) Photos of individual T-cell cultures after 6 day co-culture with tumor-pulsed DCs or with anti-CD3. Labels denote Step 1 DC conditioning treatments; Step 2 DC cultures were performed in rGMCSF+IL4, with irradiated MCA-203 cells added at 24h and CpG+LPS added at 44 hr. 4 hr later, DCs were harvested for co-culture with L-selectinlow T-cells freshly harvested from MCA-203 tumor-bearing mice. T-cells were co-cultured with DCs at a 8:1 ratio or were activated with immobilized anti-CD3. Co-cultures shown also received exogenous IL2+IL7+IL15. This is representative of three full comparison experiments, using an Olympus IX50 inverted microscope, an Olympus CPlan 10x/0.25 PhC objective lens, a Sony DSC-S85 Cybershot, and Adobe Photoshop for compilation.
Figure 4: Impacts of initial Step 1 DC conditioning treatments upon subsequent culture of T cells from tumor-bearing mice. Tumor-pulsed DC preparations and co-cultures were performed as in Fig 3b. (a) T cell fold-expansion 12 days after initial exposure to tumor-pulsed DCs or to anti–CD3; labels indicate prior DC conditioning treatments. As distinguished in the legend, the displayed T-cell expansions were performed either with no added cytokine, added rIL2 only (24 I.U./ml beginning day 2 of T-cell culture), or IL2+IL7+IL15 (50 ng IL7, 5 ng IL15). This is representative of 3 experiments. (b) Co-culture of T-cells from tumor-bearing mice with Flt3L+IL6 conditioned DCs results in superior expansion of tumor-specific T-cells. Step 1 DC conditioning was performed with Flt3L+IL6, rest of DC preparation and T cell co-cultures as in Fig 3b. After 12 day co-culture, T-cells were harvested and replated either alone or with irradiated MCA–203 or MCA–105 as stimulator cells. Monensin (Golgistop) was added after 5 hours, and cells analyzed after an additional 12 hours for intracellular IFN-γ production. Dot plots show T-cells recultured after expansion in IL2+IL7+IL15, but co-culture with tumor-pulsed, Flt3L+IL6 conditioned DCs yielded virtually identical tumor-specificity even when no cytokines were added during the 12 day coculture (not shown). B1: Dot plots display CD4 vs. IFN-γ staining; % shown in each dotplot is % of total CD4 cells producing IFN-γ. B2: Dot plots display CD8 vs. IFN-γ staining; % in each dotplot is % of total CD8 cells producing IFN-γ. This is representative of 6 experiments. (c) T-cells driven with Flt3L+IL6 conditioned DCs are highly effective as adoptive therapy. 5 day established MCA–203 subcutaneous tumors were treated i.v. with T-cells from MCA–203 bearers after 12 day culture driven by Flt3L+IL6 conditioned, tumor-pulsed DCs or by anti–CD3. Conventional nonmyelablative total body irradiation (500 cGy) was given as an adjunct prior to T-cells. Cure rates were 0/5 (A, No treatment); 2/5 (B, 5 million anti–CD3 driven T-cells; 5/5 (C, 5 million “Flt3L+IL6” DC driven T-cells); 5/5 (D, same as C but 2 million T-cells). Treatment outcome A vs C/D, p=0.008; A vs B, p=0.141; B vs C/D, p=0.04). T-cell cultures driven by Flt3L+IL6 conditioned DCs were also highly effective against more advanced tumors (Supplemental Fig 6 online). This is representative of 4 experiments.
Figure 5: In vivo fate of BM cells administered following Step 1 conditioning treatments. 18 million BM cells were labelled with CFSE and given by tail vein to mice bearing 10 day MCA-203 s.c. tumors. 48h later, mice were euthanized and single cell suspensions of tumors and spleens processed for FACS analyses.

(a) Frequency of CFSEpos cells recovered in tumor or spleen. Each bar shows average of five synchronously analyzed tumors or spleens ±sd in a single experiment for each Step 1 condition or for background staining (“No cells given”). “Flt3L+IL6” vs “Flt3L+GMCSF” or “Flt3L+IL6+GMCSF” conditioning, p<0.001.

(b) Phenotype of CFSEpos cells recovered in tumor or spleen following in vitro Flt3L+IL6 conditioning. Numbers in paretheses in each histogram display mean fluorescence specificity indices.

Results in (a) and (b) are representative of three separate experiments.
Figure 6: (a) Representative intracellular staining for pSTAT3 (pY705) vs. pSTAT5 (pY694) following various Step 1 conditioning regimens, either at end of 6 day Step 1 culture (left), or following an additional 48 hour Step 2 culture in GMCSF+IL4 (right); see details in Methods. Numbers in parentheses in each histogram display “mean fluorescence specificity indices.” Results are respectively representative of four (left) and three (right) separate experiments. (b-d) Capacities of STAT5 or STAT3 knockout mice to respond to various conditioning regimens. (b) BM was prepared from STAT5KO mice (“St5 KO”) or from littermates expressing wildtype STAT5 (“St5 LM”) and subjected to Step 1 conditioning with Flt3L+IL6 or Flt3L+IL6+GMCSF. Results shown for Flt3L+IL6+GMCSF are also highly representative of GM-CSF or Flt3L+GMCSF conditioning outcomes (not shown). Proliferation kinetics during Step 1 culture were comparable for KOs vs LMs (not shown). Histograms display mean fluorescence specificity indices for DC maturation markers following 48 hours of Step 2 culture in GMCSF+IL4, with CpG+LPS subjected to Step 1 conditioning with Flt3L+IL6 or Flt3L+IL6+GMCSF. Results shown for Flt3L+IL6+GMCSF are also highly representative of STAT3KO mice (“St3 KO”) or from LMs expressing wildtype STAT3 (“St3 LM”) and subjected to Step 1 conditioning regimens, either at end of 6 day Step 1 culture (left), or following an additional 48 hour Step 2 culture in GMCSF+IL4 (right); see details in Methods. Numbers in parentheses in each histogram display “mean fluorescence specificity indices.” Results are respectively representative of four (left) and three (right) separate experiments. (c) BM was prepared from STAT3KO mice (“St3 KO”) or from LMs expressing wildtype STAT3 (“St3 LM”) and subjected to Step 1 conditioning and Step 2 culture as in (a). Results shown for Flt3L+IL6+GMCSF are also highly representative of GM-CSF or Flt3L+GMCSF conditioning. Proliferation kinetics during Step 1 culture were comparable for KOs vs LMs, excepting that aberrant culture failure occurred when “St3 KO” BM was conditioned with Flt3L+IL6 (not shown). Results shown are representative of 2 experiments. (d) ELISA comparisons of supernatants for IL12p70 at end of Step 2 cultures. BM was obtained from KO mice or from matched wildtype LMs, placed in standard Step 1 cultures with Flt3L+IL6, Flt3L+IL6+GMCSF or GMCSF alone as conditioning agents, and finally placed in 48 hr Step 2 cultures with GMCSF+IL4, and CpG+LPS added during the final 18 hours. Culture failure was observed only when STAT3KO BM was conditioned with Flt3L+IL6. Data are presented as mean ± s.d. of 3 replicates and are representative of three (STAT5KO vs LM) or two (STAT3KO vs LM) independently performed experiments.
Figure 7:
Schematic representation of postulated STAT-dependent DC differentiation pathways. Flt3L stimulates pan-differentiation of CD34pos common myeloid and common lymphocyte precursors into CD11cpos committed DC precursors via a STAT3-dependent process. This is markedly potentiated by IL-6, but is dominantly suppressed by Step 1 exposure to GMCSF, due to inhibition of STAT3 activation and concomitant STAT5 activation (Fig 6a). Such Step 1 GMCSF exposure instead favors differentiation of CD34pos common myeloid precursors into granulocyte/monocyte progenitors (rather than committed DC precursors). The granulocyte/monocyte progenitors achieve subsequent multilineage differentiation, including STAT5-dependent differentiation into conventional DCs and macrophages, and STAT5-independent differentiation into neutrophils (Fig 6b). Phenotypically conventional DCs generated by STAT3- vs STAT5-dependent pathways differ in many critical characteristics (Fig 1-5). It should be emphasized that although Step 1 exposure to GMCSF blocks STAT3-dependent DC differentiation, Step 2 exposure of committed DC precursors to GMCSF may instead promote maturation and DC1-polarization, again by stimulating STAT5 and inhibiting STAT3 (Fig 6a).
STAT3- and STAT5-dependent pathways competitively regulate the pan-differentiation of CD34^pos^ cells into tumor-competent dendritic cells

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