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Innate immunity

Research Article

Dendritic cells modulate c-kit expression on the edge between activation and death

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Dendritic cells (DCs) are key players in immunity and tolerance. Some DCs express c-kit, the receptor for stem cell factor (SCF), nevertheless c-kit functional role and the regulation of its expression in DCs are incompletely defined. We recently demonstrated that autocrine SCF sustains a pro-survival circuit, and that SCF increases phospho-AKT in c-kit+ mouse bone marrow-derived DCs (BMdDCs). Herein we observed that CpG and PolyI:C, two stimuli mimicking bacterial and viral nucleic acids respectively, strongly inhibited c-kit expression by BMdDCs and spleen DCs in vitro and in vivo. Experiments in IFNARI$^{-/-}$ mice showed that IFN-I pathway was required for c-kit down-regulation in cDC1s, but only partially supported it in cDC2s. Furthermore, CpG and PolyI:C strongly inhibited c-kit mRNA expression. In agreement with the reduced c-kit levels, SCF pro-survival activity was impaired. Thus in the presence of exogenously provided SCF, either PolyI:C or CpG induced spleen DC death in 2 days, while at earlier times IL-6 and IL-12 production were slightly increased. In contrast, SCF improved survival of unstimulated spleen DCs expressing high c-kit levels. Our studies suggest that c-kit down-modulation is a previously neglected component of DC response to CpG and PolyI:C, regulating DC survival and ultimately tuning immune response.

Keywords: conventional dendritic cell subsets · dendritic cell survival · CpG · PolyI:C · Stem cell factor

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Introduction

Dendritic cells (DCs) are key players in immunity and tolerance [1, 2]. Under physiological conditions, DCs are in a resting or immature state, acting as quiescent sentinels distributed in healthy tissues. In the presence of pathogens and/or cell injury, DCs get

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activated, take up pathogen and cell debris and migrate to draining lymph nodes (LNs). Activated or mature DCs prime naïve T cells, thus starting adaptive responses, and orchestrate immunity by secreting several cytokines and chemokines [2].

DC activation is induced by a wide array of different pathogen- and injured cell-derived molecules (PAMPs, pathogen-associated molecular patterns and DAMPs damage-associated molecular patterns, respectively), which bind to innate immunity receptors expressed both on DC membrane and intracellularly. Innate immunity receptors include members of the Toll-Like Receptor (TLR) family, RIG-I-Like Receptor (RLR) family, etc. [3]. Members of TLR family have a variety of specificities. TLR-4, for example, recognizes LPS from Gram-negative bacteria, TLR-9 binds unmethylated CpG DNA typically present in bacterial DNA, while TLR-3 recognizes viral double strand RNA (dsRNA), as well as synthetic dsRNA, e.g. PolyI:C [3]. Moreover, dsRNA can also be sensed by MDA5 and RIG-I, both belonging to the RLR family [4], while CpG can bind to DNA-dependent kinase catalytic subunit (DNA-PKcs), another player in CpG signaling pathways in DCs [5].

DCs are highly heterogeneous [6]. At least three differentiated DC subsets are found in physiological conditions, i.e. the so-called conventional DC1s (cDC1s), conventional DC2s (cDC2s) and plasmacytoid DCs (pDCs) [6], while other subsets appear in inflammatory conditions [7]. The current view is that cDC subsets are specialized in antigen presentation to T cells, whereas pDCs in producing abundant amounts of type I Interferon (IFN-I) upon viral infection. Nevertheless, further studies are required to better define the peculiar function of each DC subset [8].

DCs undergo a limited number of divisions in the periphery and have a short life-span, being constantly replaced by newly differentiated DCs derived from the BM. For example, it is estimated that the half-life of spleen DCs is about 7 days for cDCs and shorter for pDCs [9, 10]. The two key cytokines for DC development and homeostasis are Flt3L and GM-CSF [11–13]. Additional molecules likely contribute to differentiated DC survival in lymphoid organs, as suggested by the presence of some residual cDCs in the spleen of Flt3L KO mice, and of Flt3L/GM-CSF double KO mice [12].

We recently described a previously unknown autocrine circuit driven by Stem Cell Factor (SCF) and its membrane receptor c-kit in DCs. It should be highlighted that, although c-kit is mostly recognized for its role during DC development, some differentiated DCs in lymphoid organs constitutively express c-kit, for example cDC1s and cDC2s from mouse spleen and cDC1s from mouse and human BM, while pDCs are negative for c-kit expression [2, 14]. We showed that differentiated MHC^{hi} CD40^{hi} DCs generated from mouse BM with GM-CSF (BM-derived DCs, BMdDCs) produced SCF and responded to it, e.g. by increased phosphorylation of Akt kinase. SCF silencing resulted in reduced survival of BMdDCs in vitro. Furthermore, we demonstrated that GM-CSF inhibited both SCF and c-kit expression by differentiated BMdDCs, suggesting that SCF might more prominently regulate DC survival under conditions of reduced GM-CSF availability [14]. We did not observe any effect of SCF on antigen presentation by BMdDCs, neither in MHC-I, nor in MHC-II [14].

So far, c-kit/SCF involvement in DC-mediated regulation of immune response has been demonstrated only in allergic asthma mouse models [15, 16]. It was reported that SCF/c-kit expression by DCs was essential to drive DC production of IL-6 and IL-23 in response to Cholera Toxin (CT)/Ovalbumin (OVA), ultimately controlling T cell polarization to Th2/ Th17 [15, 16]. Notably, c-kit^{+} inflammatory monocytic DCs (moDCs) were found in the lung in response to CT/OVA, and to a much minor extent in response to CpG/OVA, whereas lung DCs from healthy mice were negative for c-kit expression [15]. In vitro studies showed that c-kit mutant-lung DCs and -BMdDCs had a strongly impaired IL-6 response when stimulated with the pro-allergic stimuli CT and House Dust Mite (HDM). In contrast, IL-6 production in response to the pro-inflammatory stimulus CpG was not affected by c-kit mutation, suggesting a specific involvement of c-kit/SCF in allergic responses [16, 17]. It should not be neglected that the wild-type BMdDCs used in these experiments had extremely low levels of membrane c-kit, and strongly up-regulated it after stimulation with either CT or HDM [16]. The possibility that CpG failed to increase c-kit expression by wild-type BMdDCs was not investigated, nor the potential role played by c-kit in modulating CpG-induced IL-6 production by DCs constitutively expressing c-kit, e.g. spleen DCs.

In this article we examined the cross-talk between CpG, PolyI:C, and SCF in DC populations that constitutively express c-kit, i.e. spleen DCs and c-kit^{+} BMdDCs generated by our protocol [14]. We evaluated DC production of IL-6 and IL-12, and c-kit expression under different stimulatory conditions, both in vitro and in vivo. Our studies showed that while CpG-induced IL-6 and IL-12 production by spleen DCs were only slightly up-regulated in the presence of SCF, a strong c-kit down-regulation by spleen DCs and BMdDCs was observed in response to either CpG or PolyI:C. Spleen DCs activated by CpG and PolyI:C had a decreased survival in the presence of SCF. Thus, c-kit down-modulation by DCs responding to CpG and PolyI:C might contribute to activated DC death, possibly regulating the duration of immune response.

Results

SCF does not synergize with CpG or PolyI:C in inducing IL-6 production by BMdDCs

We used our previous protocol to obtain high numbers of BMdDCs constitutively expressing c-kit from B6 mice [14]. As shown in Fig. 1A, in a typical experiment about 94% of BMdDCs were c-kit^{+} MHC-II^{hi} CD40^{hi} cells, and only a few cells in the culture were c-kit^{-} MHC-II^{int} CD40^{int} cells. Our gating strategy and an example of CD11c staining are shown in Supporting Information Fig. 1A and B. A comprehensive analysis of our BMdDC phenotype is reported in [14]. Since the great majority of BMdDCs in our cultures were c-kit^{+}, we were able to mimic the conditions of constitutive c-kit expression, for example those of spleen DCs.

To study the possible interplay between SCF and CpG in regulating IL-6 production by c-kit^{+} DCs, we stimulated BMdDCs

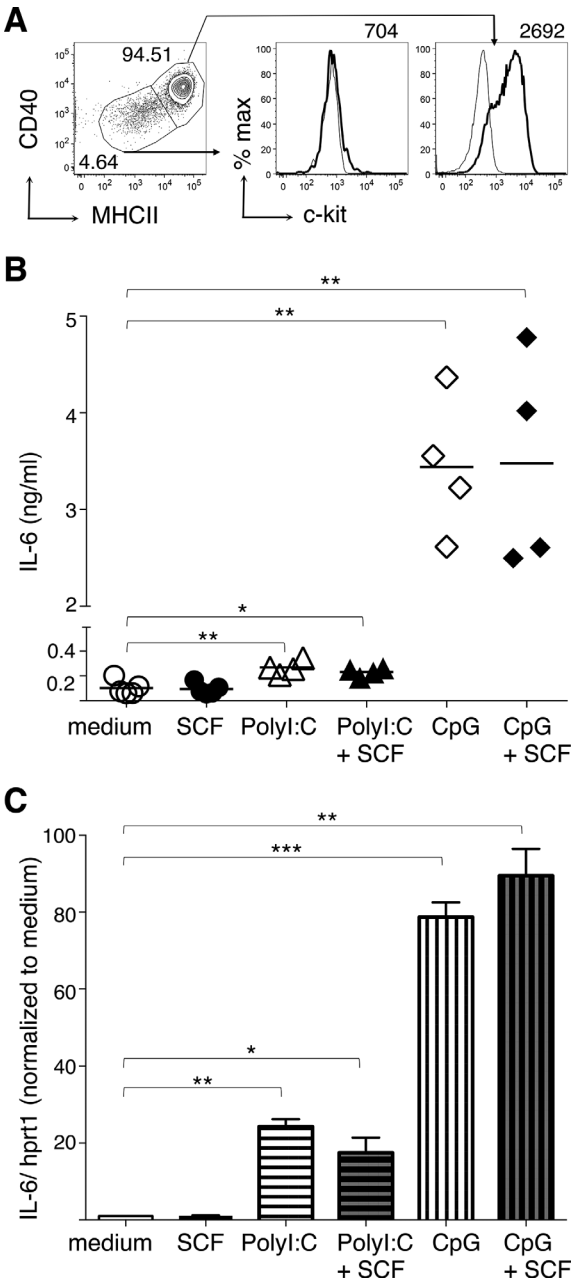

Figure 1. IL-6 production by BM-derived DCs (BMdDCs) stimulated with either CpG or PolyI:C in combination with SCF. (A) Typical flow cytometric profile of BMdDCs showing c-kit expression by MHCII^{hi} CD40^{hi} BMdDCs (see Material and Methods and Supporting Information Fig. 1). In the left panel, numbers represent percentages of cells in the indicated regions. In the histograms, thick lines represent c-kit staining profiles, thin lines isotype control mAb; c-kit Median Fluorescence Intensity (MFI) values are indicated. (B) BMdDCs (5×10^5 cells/well) were cultured in 24-well plates with medium, 50 nM CpG, 10 μg/ml PolyI:C, each tested alone or in the presence of 100 ng/mL SCF. After 24 h, supernatants were collected and IL-6 measured by ELISA in duplicates or triplicates. Individual experiment and average values (bar) are shown. (C) BMdDCs (1.2×10^6 cells/ well) were stimulated in 24-well plates as above. After 8 h, BMdDC samples were analysed by Real-Time PCR in duplicates or triplicates. IL-6 mRNA expression was calculated relative to hprt1 in arbitrary units. For each experiment, IL-6/ hprt1 levels were normalized to medium. Average values of 3 independent experiments and SD are shown. In (A), representative example of 10 N; in (B) N = 5; in (C) N = 3 (N indicates the number of independent experiments).

generated as in Fig. 1A with CpG and SCF, alone or in combination, and measured IL-6 expression by ELISA and qPCR. CpG induced a strong IL-6 response, as shown by both methods (Fig. 1B and C). IL-6 concentration in the culture supernatant was not significantly augmented when comparing CpG plus SCF with CpG alone (Fig. 1B). Although SCF slightly increased IL-6 mRNA levels induced by CpG, the difference was not statistically significant (Fig. 1C). Similar results were obtained with BMdDCs generated from Balb/c mice (Supporting Information Fig. 1C). We also tested PolyI:C, a weak IL-6 inducer, and found that IL-6 production by BMdDCs in response to PolyI:C was indeed lower than that induced by CpG, and not enhanced by SCF (Fig. 1B-C). Results were confirmed by measuring IL-6 protein concentration at graded doses of either CpG or PolyI:C, and IL-6 mRNA at different times after stimulation with CpG, alone or in combination with SCF (Supporting Information Fig. 1D and F).

CpG and PolyI:C inhibit membrane c-kit expression by BMdDCs

We then analyzed c-kit membrane expression. In agreement with our previous findings [14], we observed that BMdDCs kept in culture with medium over 1 or more days had the tendency to increase c-kit expression (compare c-kit MFI in Fig. 1A and Fig. 2A and B; plus data not shown). In contrast, c-kit membrane expression was strongly reduced after 1 day of culture with SCF, CpG or PolyI:C, each tested alone in comparison with medium (Fig. 2A and B). Combination of SCF plus PolyI:C induced a statistically higher c-kit decrease than PolyI:C alone, and a similar trend was observed with SCF plus CpG versus CpG alone (Fig. 2A and B). CpG, and to a lower extent PolyI:C, induced membrane CD40 up-regulation, that did not change in the presence of SCF. SCF alone did not induce any change in CD40 membrane expression (Fig. 2C and D). CpG-induced c-kit down-modulation was evident already after 6 h of culture, whereas that induced by PolyI:C was not (Supporting Information Fig. 2). We also used forskolin (FSK) as a positive control for c-kit up-regulation [16], and found that BMdDCs treated with FSK up-regulated c-kit membrane expression (Supporting Information Fig. 2).

CpG and PolyI:C inhibit c-kit mRNA expression by BMdDCs, and PolyI:C increases SCF mRNA expression

We performed qPCR analysis of c-kit expression by BMdDCs stimulated as above and found that CpG induced a striking decrease of c-kit mRNA compared with medium alone, with 6-fold- and 7-fold-reduced levels after 4 and 8 h of incubation respectively (Fig. 3A and B). These effects were not augmented when CpG was combined with SCF. A less pronounced decrease was induced by PolyI:C at 8 h. c-kit mRNA level was not changed in the presence of SCF alone (Fig. 3A and B). c-kit mRNA expression by BMdDCs incubated with medium for 4h and 8h was similar to that of BMdDCs at the beginning of the culture (time 0) ($p = 0.64$

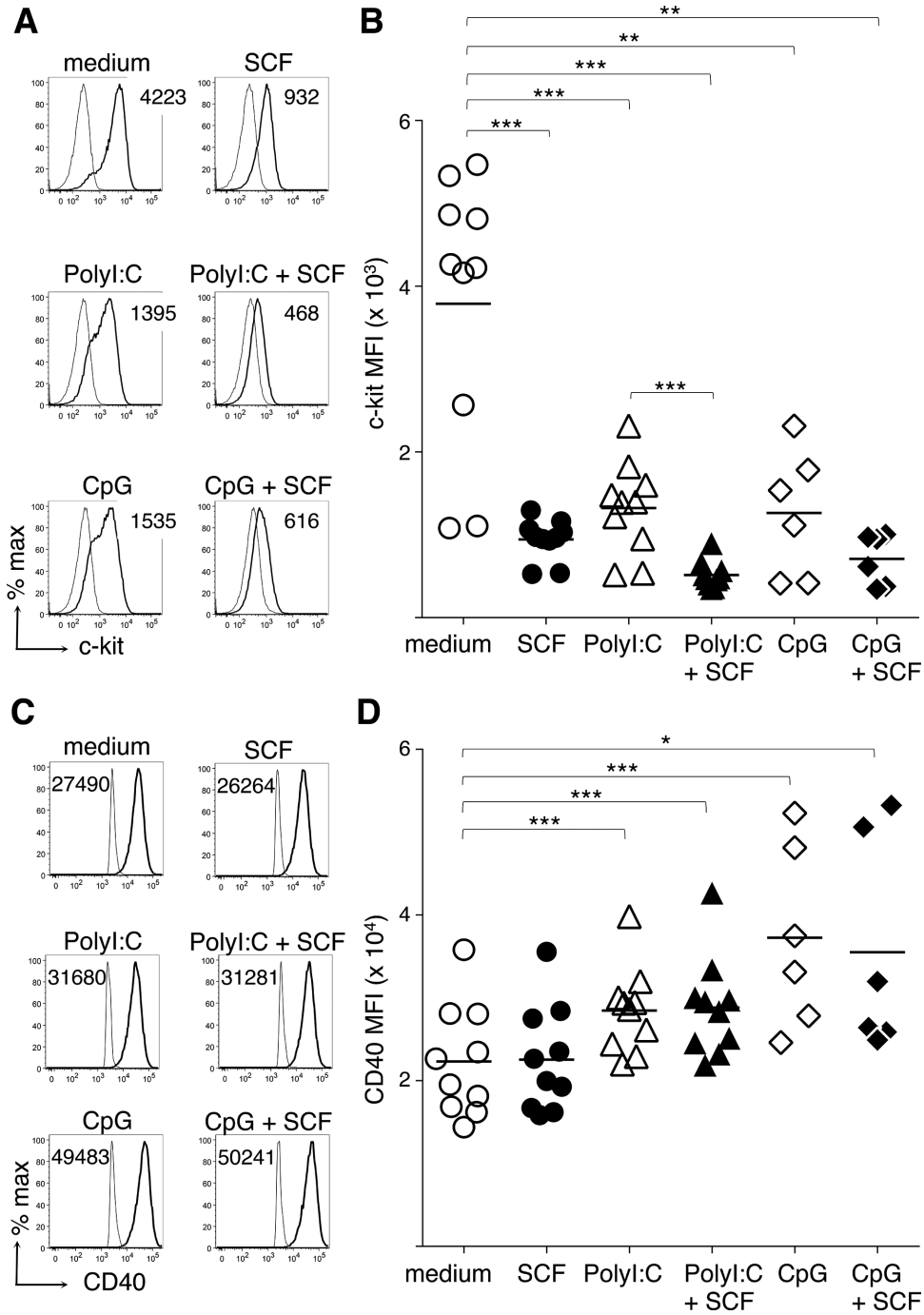

Figure 2. c-kit and CD40 membrane expression by BMdDCs stimulated with either CpG or PolyI:C in combination with SCF. BMdDCs (1.2×10^6 cells/well) were cultured in 24-well plates with medium, 50 nM CpG, 10 μg/mL PolyI:C, each tested alone or in the presence of 100 ng/mL SCF. After 24 h, cells were collected, stained with fluorochrome-conjugated mAbs and analysed by flow cytometry. (A and C) Typical histograms showing c-kit (A) and CD40 (C) expression by $MHCII^{hi}$ $CD40^{hi}$ BMdDCs, gated as in Fig. 1A. Thick lines represent c-kit (A) and CD40 (C) staining profiles, thin lines their corresponding Fluorescence Minus One (FMO) controls; c-kit (A) and CD40 (C) MFI values are indicated. (B and D) Summary of c-kit (B) and CD40 (D) MFI results. Individual experiment and average values (bar) are shown. In (A and C), representative example of 10 N; in (B and D), N = 10 (N indicates the number of independent experiments).

medium 4 h versus time 0; p = 0.46 medium 8 h versus time 0; data not shown). Furthermore, we measured SCF mRNA levels and observed that PolyI:C induced a major SCF up-regulation compared with medium, reaching a >10-fold increase at 8 h of incubation, whereas no significant change was observed with CpG (Fig. 3C and D). SCF mRNA levels were not altered by the addition of soluble SCF alone or in combination with CpG or Poly I:C (Fig. 3C and D).

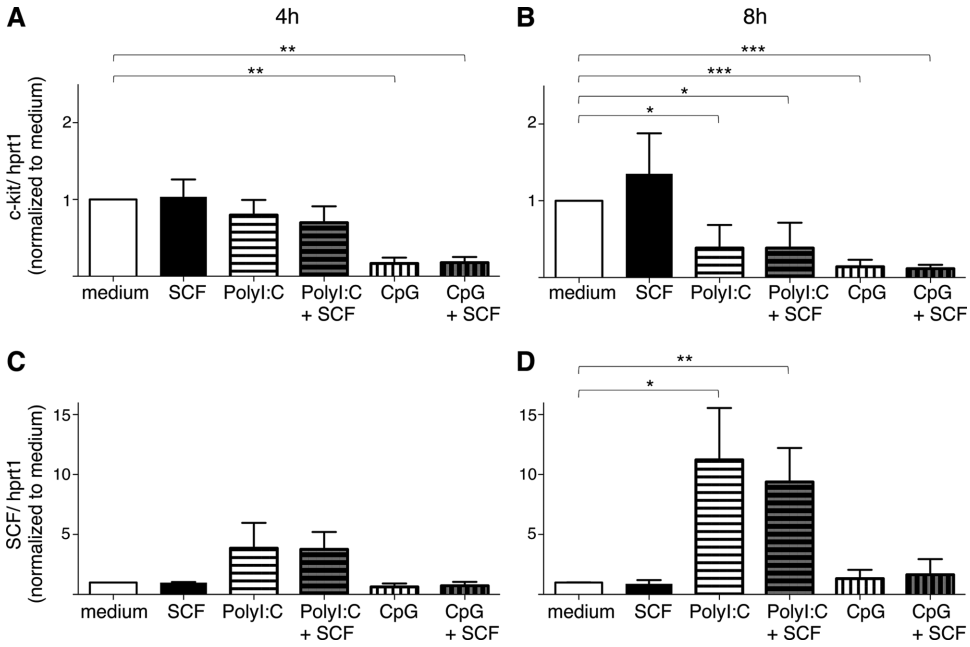

Figure 3. c-kit and SCF mRNA expression by BMdDCs stimulated with either CpG or PolyI:C in combination with SCF. BMdDCs (1.2×10^6 cells/ well) were cultured in 24-well plates with medium, 50 nM CpG, 10 μg/mL PolyI:C, each tested alone or in the presence of 100 ng/mL SCF. After 4 (A and C) and 8 (B and D) h, BMdDC samples were analysed by Real-Time PCR in triplicates. c-kit (A-B) and SCF (C-D) mRNA expression was calculated relative to hprt1 in arbitrary units; c-kit/ hprt1 levels (A-B) and SCF/ hprt1 (C-D) were normalized to corresponding medium. Average values of at least 3 independent experiments and SD are shown. In (A and C), N = representative example of 3 N; in (B and D) N = 4 (N indicates the number of independent experiments).

SCF slightly synergizes with CpG but not PolyI:C in inducing IL-6 production by spleen DCs

To investigate whether CpG and Poly I:C effects on spleen DCs were similar to those observed on BMdDCs, we purified spleen DCs with CD11c-immuno-magnetic separation from B16-Flt3L-treated B6 mice [18]. Spleen DCs purified from B16-Flt3L-treated mice constitutively expressed c-kit, similarly to spleen DCs from untreated mice (Fig. 4A, Supporting Information Fig. 3). We stimulated spleen DCs purified from B16-Flt3L-treated mice with PolyI:C and CpG in vitro, alone or in combination with SCF and measured IL-6 by ELISA (Fig. 4B). In agreement with results obtained with BMdDCs, SCF alone was unable to induce IL-6 and to modulate the weak PolyI:C-driven IL-6 production, while CpG elicited high IL-6 release. Differently from BMdDC data, a little further increase in IL-6 levels was observed when spleen DCs were stimulated with CpG plus SCF (Fig. 4B). Results were similar when graded doses of CpG were tested (Supporting Information Fig. 4A).

Furthermore, SCF had a little but statistically significant pro-survival effect on spleen DCs (Fig. 4C), in agreement with our previous results on BMdDCs [14]. To rule out that the SCF positive effect on CpG-induced IL-6 production was due to a better survival of spleen DCs, we performed intracellular staining for IL-6. The percentage of IL-6 producing cells within spleen DCs was significantly higher in the presence of CpG plus SCF than with CpG alone, being on the average 35.8, and 31.6%, respectively (Fig. 4D and E), thus confirming the small SCF synergistic effect observed by ELISA.

SCF slightly synergizes with CpG in inducing IL-12 production by spleen DCs but not by BMdDCs

We then investigated whether SCF was able to modulate IL-12 production by either BMdDCs or spleen DCs stimulated with CpG (Fig. 5). As expected, CpG was a strong IL-12 inducer while SCF alone did not stimulate IL-12 production. When used in combination with CpG, SCF slightly but significantly increased IL-12 production by spleen DCs (Fig. 5B), but not by BMdDCs (Fig. 5A), thus echoing IL-6 findings (see Fig. 1 and Fig. 4). Results obtained with Balb/c BMdDCs were similar to those obtained with B6 BMdDCs, although levels of IL-12 induced by CpG were lower (Supporting Information Fig. 4B).

CpG and PolyI:C inhibit membrane c-kit expression by spleen DCs

We then analyzed c-kit membrane expression and found that spleen DCs cultured in medium for 1 day had the tendency to increase c-kit levels, similarly to BMdDCs (compare c-kit MFI in Fig. 4A and 6A and B; plus data not shown). A strong and statistically significant c-kit down-modulation was observed after 1 day

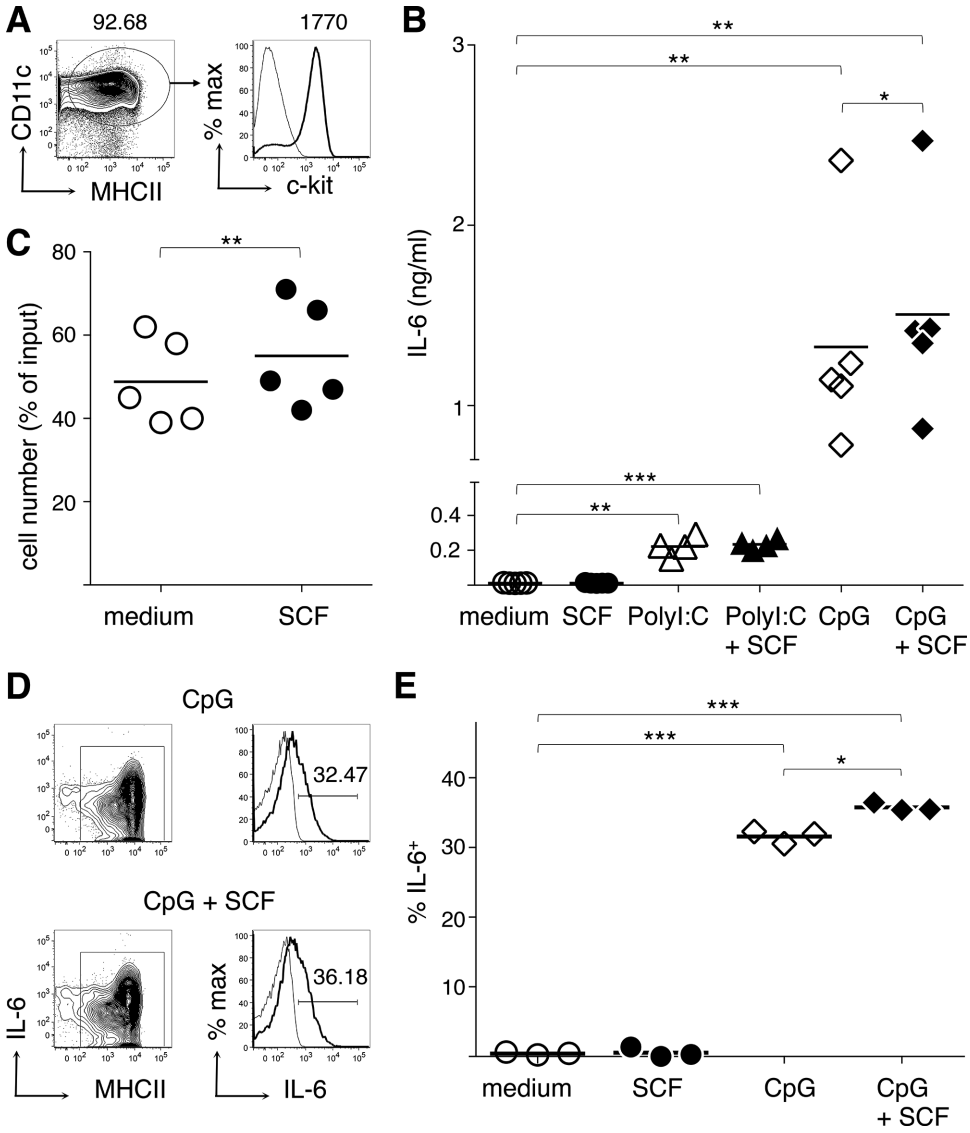

Figure 4. IL-6 production by spleen DCs stimulated with either CpG or PolyI:C in combination with SCF. (A) Typical flow cytometric profile of freshly purified spleen DCs showing c-kit expression by CD11c$^+$ MHCII$^+$ spleen DCs (see Material and Methods and Supporting Information Fig. 3B). In the left panel, numbers represent percentages of cells in the indicated region. In the histograms, thick line represents c-kit staining profiles, thin line isotype control mAb; c-kit MFI value is indicated. (B) Spleen DCs (5×10^5 cells/ well) were plated in 24-well plates with medium, 50 nM CpG, 10 μg/mL PolyI:C, each tested alone or in the presence of 100 ng/mL SCF. After 24 h of incubation, supernatants were collected and IL-6 measured by ELISA in duplicates or triplicates. (C) Spleen DCs (1.6×10^5 cells/ well) were incubated in duplicates or triplicates in 96-well plates with either medium, or 100 ng/mL SCF. At the beginning of the incubation (time 0) and after 2 days of culture, the absolute number of PI$^{(-)}$ living cells was determined by MACSQuant VYB. Data at day 2 are shown as percentage of PI$^{(-)}$ living cells at time 0 (% of input). (D-E) Spleen DCs were plated as in B. After 4 h of incubation, a cocktail containing Brefeldin A and Monensin was added to each well and cells were incubated for further 16 h. Cells were then collected and stained for intracellular IL-6. (D) Example of IL-6 staining. Numbers represent percentages of IL-6$^+$ cells in the indicated regions. (E) Summary of IL-6 results. In B, C and E individual experiment and average values (bar) are shown. In (A), representative example of 15 N; in (B), N = 5; in (C), N = 5; in (D), representative example of 3 N; in (E) N = 3 (N indicates the number of independent experiments).

of incubation with CpG or PolyI:C, resulting in a roughly 3-fold reduction of c-kit Median Fluorescence Intensity (MFI) as compared with medium (Fig. 6A and B). A similar effect was observed with SCF alone, and on the average c-kit MFI tended to be even lower in the presence of CpG plus SCF, and PolyI:C plus SCF (Fig. 6A and B). We performed additional studies culturing spleen DCs in different conditions for only 6 h, and observed that c-kit MFI with medium was 967 ± 164, with SCF 307 ± 24 ($p \leq 0.05$), and with CpG 934 ± 173 (average ± SD of 3 experiments). Thus, CpG-induced c-kit down-modulation by spleen DCs was not yet evident after only 6 h of incubation, in contrast with the findings obtained with BMdDCs (see Supporting Information Fig. 2). Notably, after 2 days of incubation with either PolyI:C or CpG in the presence of SCF, spleen DC numbers were decreased (Fig. 6C and D), suggesting that SCF was unable to efficiently support activated DC survival, possibly due to the reduced c-kit expression. Furthermore, c-kit MFI of spleen DCs incubated for 1 day with PolyI:C, CpG or medium alone positively correlated with the

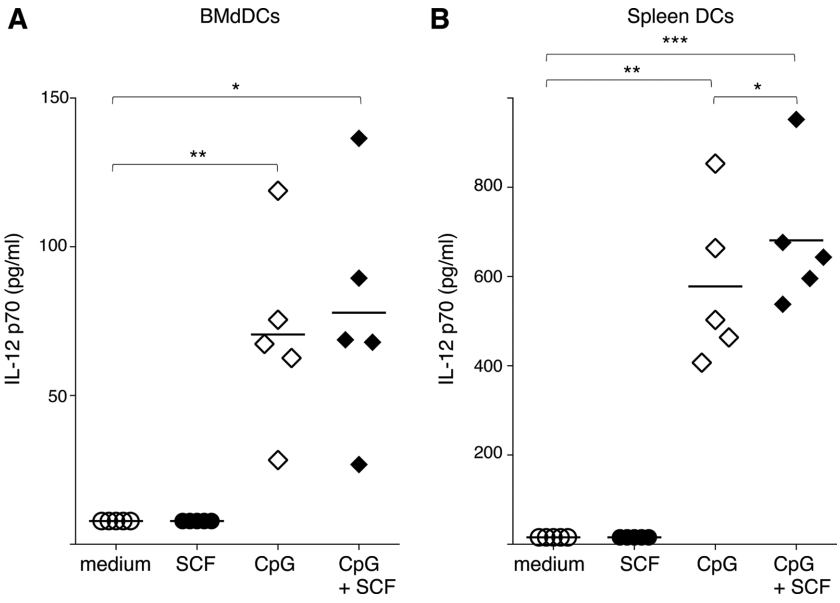

Figure 5. IL-12 production by BMdDCS and by spleen DCs stimulated with CpG in combination with SCF. BMdDCs (A) and spleen DCs (B) were cultured in 24-well plates (5×10^5 cells/ well) with medium, 50 nM CpG, 100 ng/mL SCF, and 50 nM CpG plus 100 ng/mL SCF. After 24 h, supernatants were collected and IL-12 measured by ELISA in duplicates. Individual experiment and average values (bar) are shown. In (A), N = 5; in (B), N = 5; (N indicates the number of independent experiments).

percentage of live spleen DCs in the culture (Supporting Information Fig. 4C).

Finally, we analyzed CpG and PolyI:C effects after gating on either cDC1s or cDC2s. In agreement with our previous findings on BM DCs [14], we observed that unstimulated cDC1s had a higher c-kit MFI than unstimulated cDC2s (Supporting Information Fig. 5A–C). Notably, we found that both subsets markedly down-regulated c-kit expression upon CpG- or PolyI:C-induced activation (Supporting Information Fig. 5A–C). We further observed that both cDC1s and cDC2s significantly down-regulated c-kit expression after stimulation with either TNFα or IFNβ (Supporting Information Fig. 6A and B). In contrast, LPS at 1 μg/ml did not modulate c-kit expression by cDC2s and slightly increased it by cDC1s (Supporting Information Fig. 6A and B). A

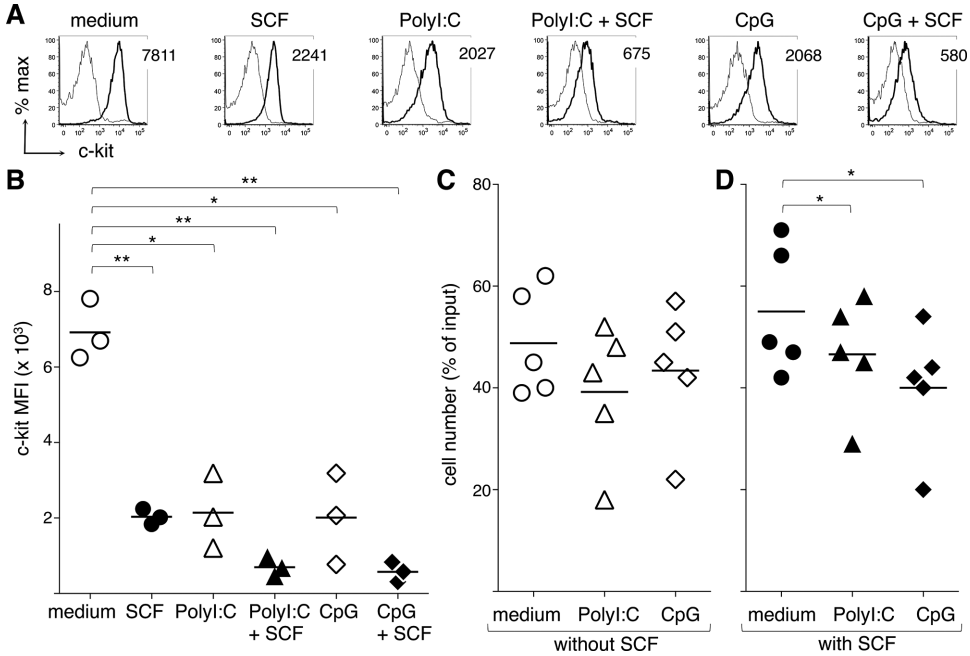

Figure 6. c-kit membrane expression and survival of spleen DCs stimulated with either CpG or PolyI:C in combination with SCF. (A-B) Spleen DCs (5×10^5 cells/ well) were cultured in 24-well plates with medium, 50 nM CpG, 10 μg/mL PolyI:C, each tested alone or in the presence of 100 ng/mL SCF. After 24 h, cells were collected, stained with fluorochrome-conjugated mAbs and analysed by flow cytometry. (A) Typical histograms showing c-kit expression by $CD11c^+$ $MHCII^+$ spleen DCs, gated as in Fig. 4A. Thick lines represent c-kit staining profiles, thin lines FMO controls; c-kit MFI values are indicated. (B) Summary of c-kit results. (C-D) Spleen DCs were cultured as in fig. 4C with medium, 50 nM CpG, 10 μg/mL PolyI:C, each tested alone or in the presence of 100 ng/mL SCF. After 2 days, the absolute number of $PI^{(-)}$ living cells was determined as in fig. 4C. In B-D individual experiment and average values (bar) are shown. In (A), representative example of 3 N; in (B), N = 3; in (C-D), N = 5 (N indicates the number of independent experiments).

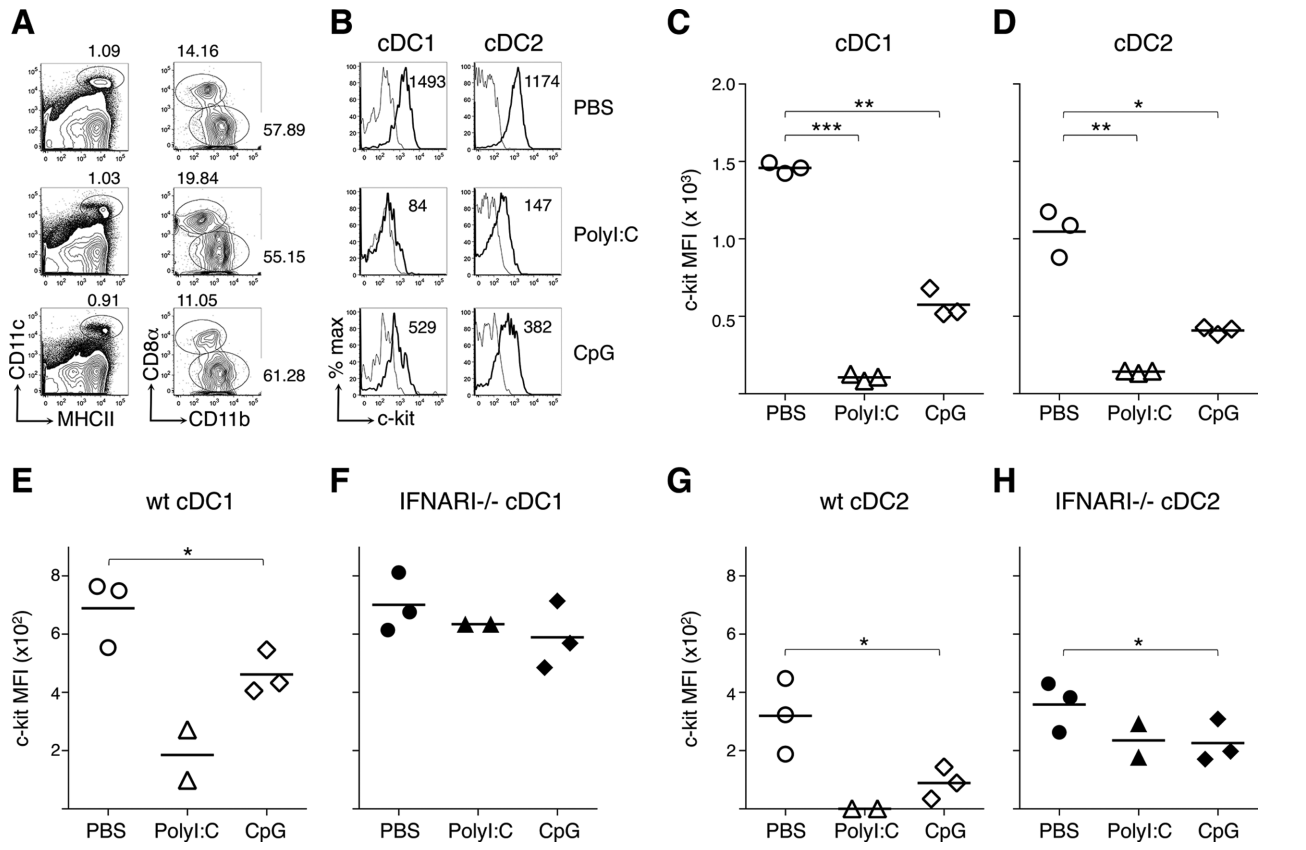

Figure 7. c-kit membrane expression by spleen cDC1s and cDC2s after in vivo treatment with either CpG or PolyI:C of wild-type and IFNARI$^{-/-}$ mice. Mice were intraperitoneally injected with either 150 μg PolyI:C or 2.5 nmoles CpG, or else PBS as a control. After 24 h, spleen cells were obtained, stained with fluorochrome-conjugated mAbs and analysed by flow cytometry. (A) Flow cytometric profiles of spleen cells from B6 mice treated as indicated. In the left panels, numbers represent percentages of CD11c$^+$ MHCII$^+$ DCs in the indicated regions; in the right panels, percentages of CD8α$^+$ cDC1s and CD11b$^+$ cDC2s in the indicated regions. (B) c-kit histograms of CD8α$^+$ cDC1s and CD11b$^+$ cDC2s from B6 mice, gated as in A. Thick lines represent c-kit staining profiles, thin lines isotype control mAb; c-kit MFI values are indicated. (C-D) Summary of c-kit results in cDC1s (C) and cDC2s (D) from B6 mice. Individual mice and average values (bar) are shown. (E-H) Summary of c-kit results in cDC1s (E-F) and cDC2s (G-H) from male IFNARI$^{-/-}$ mice and wild-type (wt) littermates. Individual mice and average values (bar) are shown. In (A-B), representative example of individual mice from 2 independent experiments; in (C-D), 9 mice examined in 2 independent experiments; in (E-H) 16 mice examined in 3 independent experiments; in each experiment 2–3 IFNARI$^{-/-}$ mice and 2–3 wt mice were examined in parallel.

similar trend was found at 5 μg/ml LPS (N = 2 experiments, data not shown). Since IFNβ was able to directly inhibit c-kit expression, CpG and PolyI:C could possibly act indirectly on c-kit expression by stimulating type I IFN production.

CpG and PolyI:C inhibit membrane c-kit expression by spleen DCs in vivo

We then analyzed the effects of CpG and PolyI:C on c-kit membrane expression by spleen cDC subsets in vivo. To this aim, normal B6 mice were injected intraperitoneally with either PolyI:C or CpG, or else PBS as a control. After 1 day, mice were killed and spleen cells were analyzed by flow cytometry. Fig. 7A-B show representative flow cyometric profiles, and fig. 7C-D a summary of results. In vivo treatment with CpG or PolyI:C induced a statistically significant c-kit down-regulation by cDC1s, as well as by cDC2s (Fig. 7C and D), thus confirming our in vitro results.

As a control for spleen DC activation, we also checked MHCII, CD40 and CD11c membrane expression by CD11c$^+$ MHCII$^+$ DCs and found that CD40 MFI was significantly up-regulated after treatment with either CpG or PolyI:C, MHCII significantly up-regulated with PolyI:C and CD11c significantly down-regulated with CpG [19] (Table 1).

Table 1. MHCII, CD11c and CD40 expression by CD11c$^+$ MHCII$^+$ spleen DCs from B6 mice injected with PolyI:C, CpG and PBS

	MHCII MFI	CD40 MFI	CD11c MFI
PBS	10523 ± 4274	526 ± 39	31474 ± 2272
PolyI:C	15222 ± 3159 (*)	1563 ± 270 (*)	21157 ± 3598
CpG	9956 ± 1934	1104 ± 65 (**)	22155 ± 943 (**)

Spleen cells were obtained from B6 mice treated as in Fig. 7, stained with fluorochrome-conjugated mAbs and analysed by flow cytometry. Numbers represent average values ± SD of MHCII, CD40 and CD11c MFI of CD11c$^+$ MHCII$^+$ spleen DCs, gated as in Fig. 7A. Summary of results obtained with 9 mice in 2 independent experiments (same mice as in Fig. 7C and D).

To investigate whether CpG and PolyI:C in vivo effects were mediated by type I IFN, we took advantage of IFNARI$^{-/-}$ mice

[20]. We observed that CpG significantly inhibited c-kit expression by spleen cDC2s but not by spleen cDC1s from IFNARI$^{-/-}$ mice. The CpG-triggered negative regulation of c-kit expression by spleen cDC2s was less pronounced in IFNARI$^{-/-}$ mice than in control mice. A similar trend was observed for c-kit down-regulation induced by PolyI:C (Fig. 7E–H).

Discussion

We showed here that PolyI:C and CpG inhibited c-kit expression by both spleen cDC1s and cDC2s, not only in vitro but also in vivo. Furthermore, TNFα and IFNβ inhibited c-kit expression by both spleen cDC subsets, while LPS did not, and even induced a slight c-kit up-regulation by cDC1s. In vivo experiments in IFNRAI$^{-/-}$ mice showed that IFNAR expression was required for CpG- and PolyI:C-triggered inhibitory effect in cDC1s but not in cDC2s. It is possible that LPS had a reduced ability to induce type I IFN in comparison with CpG and PolyI:C, or induced it with a different kinetics [21]. The molecular bases for the differences between cDC1s and cDC2s remain to be determined. In this context, it is intriguing that RelB specifically controls differentiation of a distinct population of c-kit^{+} CD172^{+} DCs in the spleen that preferentially induces Th2 response [22], and that the pro-allergic stimuli CT and HDM are able to increase membrane c-kit expression by BMdDCs in vitro and to recruit c-kit^{+} inflammatory moDCs into the lung [15, 16]. Taken together, these results point to c-kit as a potential surface marker to discriminate between different kinds of DC activation, possibly supporting distinct types of polarized responses. Future studies on c-kit modulation by a wider array of stimuli will be necessary to test this possibility.

Using BMdDCs expressing high levels of c-kit generated with our previous protocol [14], we showed a significant reduction of c-kit mRNA after 4 and 8 h of stimulation with CpG, and after 8 h of incubation with PolyI:C. The rapid effect of CpG on c-kit mRNA was reflected by reduced c-kit membrane expression, as measured by flow cytometry after 6 h of stimulation. After 24 h of incubation, both CpG and PolyI:C inhibited membrane c-kit expression. Although high levels of autocrine SCF induced by PolyI:C could possibly contribute to membrane c-kit down-regulation, our qPCR results showed that c-kit expression was strongly inhibited at the mRNA level. Furthermore, CpG also inhibited membrane c-kit expression by spleen DCs, but with a slow kinetics, as CpG effect was evident after 24 but not yet after 6 h of treatment.

Exogenously provided SCF had no effect on IL-6 and IL-12 production when tested alone, and it did not modulate PolyI:C-induced IL-6 production. In contrast, spleen DCs produced a slightly higher amount of IL-6 when stimulated with CpG plus SCF, as compared with CpG alone. Results were statistically significant and similar by ELISA and intracellular staining. This minor but reproducible synergy was not observed in BMdDCs. The difference between the two types of DCs might be explained by the faster kinetics of c-kit inhibition observed in CpG-stimulated BMdDCs. Along the same line, spleen DCs but not BMdDCs, produced a slightly higher amount of IL-12 when stimulated for 1 day with CpG plus SCF, as compared with CpG alone. Results were similar with BMdDCs generated from either B6 or Balb/c mice, although —as expected [23]—the former produced higher amounts of IL-12 than the latter.

Taken together, our results suggest that c-kit down-regulation dominates the response to CpG and PolyI:C, partially or completely inhibiting the possibility of a synergistic effect of SCF on CpG-driven IL-6 and IL-12 production. In brief, our results suggest that c-kit signalling —while being a positive regulator of allergy [16, 17]— is inhibited via reduced mRNA and membrane protein expression during DC response to the proinflammatory stimuli PolyI:C and CpG.

Incubation with exogenously provided SCF for 2 days had a little but statistically significant protective effect against spontaneous spleen DC death in vitro, in agreement with our previous findings showing that autocrine SCF supports BMdDC survival [14]. We also measured SCF protein expression by spleen DCs after 1 day of culture with medium and found that cell lysates contained 281.8 ± 97.9 pg/ml of SCF by ELISA assay [14]. Thus, our results on spleen DCs show that not only these cells produce autocrine SCF, but they also respond to exogenously provided SCF by increased survival. Nevertheless, in the presence of SCF spleen DC absolute numbers were reduced after 2 days of stimulation with PolyI:C or CpG. Taken together, our results suggest that interruption of SCF/c-kit pro-survival circuit due to reduced c-kit expression might contribute to DC death following DC maturation induced by CpG and PolyI:C [24–26].

We previously showed that SCF signaling in BMdDCs increases AKT phosphorylation [14]. This pathway is likely involved in SCF pro-survival effects in DCs, and through the activation of NF-κB, in the slight synergistic effect of SCF on CpG-induced IL-6 and IL-12 production by spleen DCs [27, 28]. TLRs signal through macromolecular complexes [29], and it has been shown that tyrosine phosphorylation plays a role in TLR-mediated activation pathway [30]. It remains to be determined whether the observed CpG-SCF interplay involves tyrosine phosphorylation events mediated by c-kit kinase activity, but this is beyond the scope of our study.

It is becoming more and more evident that DC homeostasis is regulated at multiple levels, both intrinsic and extrinsic to the cell. Our results suggest a role for the SCF/c-kit axis in tuning DC homeostasis after activation, with potential implications in translational medicine. For example, a better knowledge of the SCF/c-kit pathway might be helpful to counteract the detrimental depletion of DCs that occurs in patients with sepsis [31] and contributes to immunosuppression and worsening of the disease [32]. Furthermore, it is intriguing that a recent transcriptomic analysis of tumor infiltrating DCs in human samples showed that c-kit is highly correlated with a better prognosis [33]. This aspect deserves further studies, considering the growing evidence that transcriptomic analysis of tumor microenvironment, including DCs in tumor associated tertiary lymphoid structures, is instrumental to appropriately guide prognosis and therapy of human cancers [34].

Material and methods

Cytokines, reagents and culture media

Recombinant mouse SCF and TNFα were purchased from Immunotools (Friesoythe, Germany), recombinant mouse GM-CSF from Peprotech (Rocky Hill, NJ, USA), and recombinant mouse IFNβ from PBL Assay Science (Piscataway, NJ, USA). Opti-MEM Medium (ThermoFisher Scientific, Waltham, MA, USA) was supplemented with Glutamine, penicillin/streptomycin, 50 μM β-Mercaptoethanol (medium). This medium was used for all the experiments, except for the 7-day culture of BM cells with GM-CSF (see below). RPMI Medium 1640 (Sigma-Aldrich, Milan, Italy) was supplemented as above, plus 10% Heat-Inactivated Fetal Calf Serum (FCS) (Complete RPMI medium). LPS from *E. coli* was purchased from Enzo Life Sciences (Farmingdale, NY, USA), high molecular weight Poly I:C (Tlrl-pic- 5) and Class B CpG oligonucleotide 1668 (Tlrl-1668, sequence 5-TCCATGACGTTCCTGATGCT-3') from Invivogen (San Diego, California, USA). Forskolin was purchased from Sigma-Aldrich.

Mouse treatment and DC preparation

Female C57BL/6J (B6) and Balb/C mice purchased from Charles River (Calco, Italy), and 129 Sv IFNARI$^{-/-}$ (IFNARI$^{-/-}$) mice [20] were housed at the animal facility of Istituto Superiore di Sanità of Rome (ISS), according to institutional guidelines (DL116/92 and 26/2014).

To generate BMdDCs, mice were sacrificed at 5–16 weeks of age and BM was obtained from tibias and femurs [35, 36]. c-kit^{+} DCs were generated from BM cells as we previously described [14]. Briefly, BM cells were cultured in Complete RPMI medium with GM-CSF. At day 7, we collected non-adherent and slightly adherent cells after detachment with PBS 3 mM EDTA. CD11c^{+} cells were highly purified (≥99% pure) with anti-CD11c magnetic microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and re-plated for 2 days in Opti-MEM medium supplemented as above (medium), in the presence of 20 ng/mL of GM-CSF. At day 9, BMdDCs containing, at an average, ≥91% c-kit^{+} MHC-II^{hi} CD40^{hi} cells were obtained and used for experiments.

To obtain high number of spleen DCs, B6 mice were injected subcutaneously into the flanks with 2×10^{6} B16-Flt3L cells [18]. After 8 days, mice were killed, and CD11c^{+} cells purified from spleen cell suspension with anti-CD11c magnetic microbeads (Miltenyi Biotec). We thus obtained spleen DC preparations containing, at an average, ≥94% CD11c^{+} MHCII^{+} DCs.

For the studies to evaluate the in vivo effects of PolyI:C and CpG, B6 mice, IFNARI$^{-/-}$ and age- and sex-matched littermate controls were injected intraperitoneally with either 150 μg of PolyI:C, or 2.5 nmol of CpG, or else PBS as a control. After 1 day, mice were killed and spleen cells were analyzed.

Membrane and intracellular staining and flow cytometry

Cell membrane staining was performed with fluorochrome-conjugated monoclonal antibodies (mAbs), after blocking with anti-FcγR (clone 2.4G2) mAb. The following mAbs were used (clone indicated in parentheses): anti-CD11c (HL3), anti-I-Ab or MHCII (M5/114.15.2) anti-CD40 (HM40-3), anti-CD11b (M1/70), anti-CD8α (53-6.7), anti-c-kit (2B8) (from BD Biosciences; Biolegend, San Diego, CA, USA; Miltenyi Biotec; eBioscience, ThermoFisher Scientific; conjugated with FITC, phycoerythrin (PE), PE-Cy7, APC, APC-Vio770). Dead cells were excluded with Propidium Iodide (PI, Sigma-Aldrich). Intracellular staining for IL-6 was performed following cell stimulation in the presence of Brefeldin A and Monensin (Protein Transport Inhibitor Cocktail, eBioscience, ThermoFisher Scientific). After membrane staining, cells were fixed, permeabilized and stained using either anti-IL-6 mAb PE (clone MP5-20F3) or isotype control PE (clone RTK2071) (both from Biolegend). Samples were analysed by FACSCanto I and II (BD Biosciences). Data were analysed using FlowJo software, v.9.7.6 (FlowJo, Ashland, OR, USA).

Real-time PCR

Total RNA was extracted from BMdDCs and spleen DCs using the TriReagent (Sigma-Aldrich). cDNA was synthesized from 1 μg of total RNA using M-MLV Reverse Transcriptase (Promega, Madison, WI, USA), according to the manufacturer's instructions. Real-time PCR was performed using TaqMan Gene expression Assays and the ABI Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA, USA). cDNAs were amplified with primers for IL-6 (Mm00446190_m1), c-kit (Mm00445212_m1), SCF (Mm00442972_m1), and hprt-1 (Mm00446968_m1) (Applied Biosystems, ThermoFisher Scientific), all conjugated with fluorochrome FAM. Relative expression of each gene versus hprt-1 was calculated according to the $2^{-\Delta\Delta ct}$ method.

ELISA

Spleen DCs and BMdDCs (both at 5×10^{5} cells/ well) were cultured in different conditions. After 24 h, supernatants were collected. IL-6 was tested by Mouse IL-6 Quantikine ELISA kit (R&D-System, Minneapolis, USA), and IL-12 by Mouse IL-12p70 Quantikine ELISA kit (R&D-System).

Cell number determination

Spleen DCs (1.6×10^{5} cells/ well) were plated in 96-well plates in different conditions. At the start of the culture (time 0), and after 2 days of incubation, spleen DC absolute number was determined

by MACSQuant VYB instrument (Miltenyi Biotec), after dead cell exclusion by PI. Data were analysed using FlowJo software, v.10.2.

Statistics

Each experimental group was compared with its corresponding control group (i.e. medium, or PBS) by performing a two-tailed paired Student's *t*-test. Two-tailed paired Student's t-test was also used to compare PolyI:C versus PolyI:C + SCF, and CpG versus CpG + SCF. Student's t-tests and Spearman's correlation test were performed using Prism v.6.0f, GraphPad Software (La Jolla, CA, USA). Differences were considered significant when $*p \leq 0.05$; $**p \leq 0.01$; $***p \leq 0.001$.

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References

1 **Zanoni, I. and Granucci, F.**, The regulatory role of dendritic cells in the induction and maintenance of T-cell tolerance. *Autoimmunity* 2011. **44**: 23–32.

2 **Merad, M., Sathe, P., Helft, J., Miller, J. and Mortha, A.**, The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting. *Annu Rev Immunol* 2013. **31**: 563–604.

3 **Akira, S., Uematsu, S. and Takeuchi, O.**, Pathogen recognition and innate immunity. *Cell* 2006. **124**: 783–801.

4 **Kato, H., Takeuchi, O., Sato, S., Yoneyama, M., Yamamoto, M., Matsui, K., Uematsu, S. et al.**, Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* 2006. **441**: 101–105.

5 **Ma, C., Muranyi, M., Chu, C. H., Zhang, J. and Chu, W. M.**, Involvement of DNA-PKcs in the IL-6 and IL-12 response to CpG-ODN is mediated by its interaction with TRAF6 in dendritic cells. *PLoS One* 2013. **8**: e58072

6 **See, P., Dutertre, C. A., Chen, J., Gunther, P., McGovern, N., Irac, S. E., Gunawan, M. et al.**, Mapping the human DC lineage through the integration of high-dimensional techniques. *Science* 2017. **356**

7 **Guilliams, M., Ginhoux, F., Jakubzick, C., Naik, S. H., Onai, N., Schraml, B. U., Segura, E. et al.**, Dendritic cells, monocytes and macrophages: a unified nomenclature based on ontogeny. *Nat Rev Immunol* 2014. **14**: 571–578.

8 **Mildner, A. and Jung, S.**, Development and function of dendritic cell subsets. *Immunity* 2014. **40**: 642–656.

9 **Liu, K., Waskow, C., Liu, X., Yao, K., Hoh, J. and Nussenzweig, M.**, Origin of dendritic cells in peripheral lymphoid organs of mice. *Nat Immunol* 2007. **8**: 578–583.

10 **Waskow, C., Liu, K., Darrasse-Jeze, G., Guermonprez, P., Ginhoux, F., Merad, M., Shengelia, T. et al.**, The receptor tyrosine kinase Flt3 is required for dendritic cell development in peripheral lymphoid tissues. *Nat Immunol* 2008. **9**: 676–683.

11 **Vremec, D., Lieschke, G. J., Dunn, A. R., Robb, L., Metcalf, D. and Shortman, K.**, The influence of granulocyte/macrophage colony-stimulating factor on dendritic cell levels in mouse lymphoid organs. *Eur J Immunol* 1997. **27**: 40–44.

12 **Kingston, D., Schmid, M. A., Onai, N., Obata-Onai, A., Baumjohann, D. and Manz, M. G.**, The concerted action of GM-CSF and Flt3-ligand on in vivo dendritic cell homeostasis. *Blood* 2009. **114**: 835–843.

13 **van de Laar, L., Coffer, P. J. and Woltman, A. M.**, Regulation of dendritic cell development by GM-CSF: molecular control and implications for immune homeostasis and therapy. *Blood* 2012. **119**: 3383–3393.

14 **Barroeta Seijas, A. B., Simonetti, S., Vitale, S., Runci, D., Quinci, A. C., Soriani, A., Criscuoli, M. et al.**, GM-CSF Inhibits c-Kit and SCF Expression by Bone Marrow-Derived Dendritic Cells. *Front Immunol* 2017. **8**: 147

15 **Oriss, T. B., Krishnamoorthy, N., Raundhal, M., Morse, C., Chakraborty, K., Khare, A., Huff, R. et al.**, Cutting Edge: MMP-9 inhibits IL-23p19 expression in dendritic cells by targeting membrane stem cell factor affecting lung IL-17 response. *J Immunol* 2014. **192**: 5471–5475.

16 **Krishnamoorthy, N., Oriss, T. B., Paglia, M., Fei, M., Yarlagadda, M., Vanhaesebroeck, B., Ray, A. et al.**, Activation of c-Kit in dendritic cells regulates T helper cell differentiation and allergic asthma. *Nat Med* 2008. **14**: 565–573.

17 **Oriss, T. B., Krishnamoorthy, N., Ray, P. and Ray, A.**, Dendritic cell c-kit signaling and adaptive immunity: implications for the upper airways. *Curr Opin Allergy Clin Immunol* 2014. **14**: 7–12.

18 **Mach, N., Gillessen, S., Wilson, S. B., Sheehan, C., Mihm, M. and Dranoff, G.**, Differences in dendritic cells stimulated in vivo by tumors engineered to secrete granulocyte-macrophage colony-stimulating factor or Flt3-ligand. *Cancer Res* 2000. **60**: 3239–3246.

19 **Singh-Jasuja, H., Thiolat, A., Ribon, M., Boissier, M. C., Bessis, N., Rammensee, H. G. and Decker, P.**, The mouse dendritic cell marker CD11c is down-regulated upon cell activation through Toll-like receptor triggering. *Immunobiology* 2013. **218**: 28–39.

20 **Muller, U., Steinhoff, U., Reis, L. F., Hemmi, S., Pavlovic, J., Zinkernagel, R. M. and Aguet, M.**, Functional role of type I and type II interferons in antiviral defense. *Science* 1994. **264**: 1918–1921.

21 **Hassanzadeh-Kiabi, N., Yáñez, A., Dang, I., Martins, G. A., Underhill, D. M. and Goodridge, H. S.**, Autocrine Type I IFN Signaling in Dendritic Cells Stimulated with Fungal β-Glucans or Lipopolysaccharide Promotes CD8 T Cell Activation. *The Journal of Immunology* 2017. **198**: 375–382.

22 **Andreas, N., Riemann, M., Castro, C. N., Groth, M., Koliesnik, I., Engelmann, C., Sparwasser, T. et al.**, A new RelB-dependent $CD117^+$ $CD172a^+$

murine DC subset preferentially induces Th2 differentiation and supports airway hyperresponses in vivo. *Eur J Immunol* 2018. **48**: 923–936.

23 **Liu, T., Matsuguchi, T., Tsuboi, N., Yajima, T. and Yoshikai, Y.**, Differences in Expression of Toll-Like Receptors and Their Reactivities in Dendritic Cells in BALB/c and C57BL/6 Mice. *Infection and Immunity* 2002. **70**: 6638–6645.

24 **Zanoni, I. and Granucci, F.**, Regulation of antigen uptake, migration, and lifespan of dendritic cell by Toll-like receptors. *J Mol Med (Berl)* 2010. **88**: 873–880.

25 **Fuertes Marraco, S. A., Scott, C. L., Bouillet, P., Ives, A., Masina, S., Vremec, D., Jansen, E. S. et al.**, Type I interferon drives dendritic cell apoptosis via multiple BH3-only proteins following activation by PolyIC in vivo. *PLoS One* 2011. **6**: e20189

26 **Zou, J., Kawai, T., Tsuchida, T., Kozaki, T., Tanaka, H., Shin, K. S., Kumar, H. et al.**, Poly IC triggers a cathepsin D- and IPS-1-dependent pathway to enhance cytokine production and mediate dendritic cell necroptosis. *Immunity* 2013. **38**: 717–728.

27 **Liang, J., Wu, Y. L., Chen, B. J., Zhang, W., Tanaka, Y. and Sugiyama, H.**, The C-kit receptor-mediated signal transduction and tumor-related diseases. *Int J Biol Sci* 2013. **9**: 435–443.

28 **Oeckinghaus, A., Hayden, M. S. and Ghosh, S.**, Crosstalk in NF-κB signaling pathways. *Nat Immunol* 2011. **12**: 695–708.

29 **Bryant, C. E., Symmons, M. and Gay, N. J.**, Toll-like receptor signalling through macromolecular protein complexes. *Mol Immunol* 2015. **63**: 162–165.

30 **Chattopadhyay, S. and Sen, G. C.**, Tyrosine phosphorylation in Toll-like receptor signaling. *Cytokine Growth Factor Rev* 2014. **25**: 533–541.

31 **Hotchkiss, R. S., Tinsley, K. W., Swanson, P. E., Grayson, M. H., Osborne, D. F., Wagner, T. H., Cobb, J. P. et al.**, Depletion of Dendritic Cells, But Not Macrophages, in Patients with Sepsis. *The Journal of Immunology* 2002. **168**: 2493–2500.

32 **Hotchkiss, R. S., Monneret, G. and Payen, D.**, Sepsis-induced immunosuppression: from cellular dysfunctions to immunotherapy. *Nat Rev Immunol* 2013. **13**: 862–874.

33 **Broz, M. L., Binnewies, M., Boldajipour, B., Nelson, A. E., Pollack, J. L., Erle, D. J., Barczak, A. et al.**, Dissecting the tumor myeloid compartment reveals rare activating antigen-presenting cells critical for T cell immunity. *Cancer Cell* 2014. **26**: 638–652.

34 **Goc, J., Germain, C., Vo-Bourgais, T. K., Lupo, A., Klein, C., Knockaert, S., de Chaisemartin, L. et al.**, Dendritic cells in tumor-associated tertiary lymphoid structures signal a Th1 cytotoxic immune contexture and license the positive prognostic value of infiltrating CD8+ T cells. *Cancer Res* 2014. **74**: 705–715.

35 **Parretta, E., Cassese, G., Barba, P., Santoni, A., Guardiola, J. and Di Rosa, F.**, CD8 cell division maintaining cytotoxic memory occurs predominantly in the bone marrow. *J Immunol* 2005. **174**: 7654–7664.

36 **Quinci, A. C., Vitale, S., Parretta, E., Soriani, A., Iannitto, M. L., Cippitelli, M., Fionda, C. et al.**, IL-15 inhibits IL-7Ralpha expression by memory-phenotype CD8(+) T cells in the bone marrow. *Eur J Immunol* 2012. **42**: 1129–1139.

Abbreviations: **BM**: Bone Marrow · **BMdDC**: bone-marrow derived DC · **cDC1**: conventional DC1 · **cDC2**: conventional DC2 · **DC**: dendritic cell · **LNs**: lymph nodes · **MFI**: Median Fluorescence Intensity · **pDC**: plasmacytoid DC · **SCF**: Stem Cell Factor

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