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Peripheral blood CD56\textsuperscript{bright} NK cells respond to stem cell factor and adhere to its membrane-bound form after upregulation of c-kit

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CD56\textsuperscript{bright} NK cells express receptors for IL-2, IL-7, IL-15, and SCF. We found that human peripheral blood CD56\textsuperscript{bright} NK cells responded to IL-2, IL-7, IL-15 by phosphorylating STAT-5, ERK, and Akt but did not respond to SCF. However, CD56\textsuperscript{bright} NK cells in culture upregulated c-kit transcription three to fourfold, which led to a steady increase in c-kit and a concomitant acquisition of responsiveness to SCF. After 44 h, CD56\textsuperscript{bright} NK cells had upregulated c-kit approximately 20-fold and phosphorylated ERK and Akt in response to SCF concentrations well below levels present in plasma. CD56\textsuperscript{bright} NK cells cultured in IL-15 maintained c-kit transcription-expression at ex vivo levels and did not become responsive to SCF. Furthermore, SCF-responsive, CD56\textsuperscript{bright}c-kit\textsuperscript{high} NK cells swiftly downregulated c-kit and stopped responding to SCF after IL-15 stimulation. However, commitment of CD56\textsuperscript{bright} NK cells to a c-kit-negative, SCF-unresponsive state did not occur, as after 5 days of culture, withdrawal of IL-15 restored c-kit to maximal levels and reestablished SCF-responsiveness. CD56\textsuperscript{bright} NK cells that had upregulated c-kit firmly adhered to COS cells transfected with the membrane form of SCF. Furthermore, SCF signaling significantly increased the capacity of CD56\textsuperscript{bright} NK cells to degranulate. Collectively, our data suggest that c-kit on human CD56\textsuperscript{bright} NK cells is a functional receptor that is downregulated in peripheral blood, possibly to render CD56\textsuperscript{bright} NK cells unresponsive to the SCF therein.

Keywords: c-Kit · Cytokines · Human · Inflammation · NK cells · SCF

Introduction

NK cells are involved in the early response to pathogens as well as in the recognition of autologous cells under stress resulting from infection or transformation. In humans, they are identified on the basis of expression of CD56 and absence of CD3. NK cells consist of at least two subpopulations [1, 2]. One population expresses relatively low levels of CD56 (CD56\textsuperscript{dim}) and appears to be end-stage effector cells endowed with a strong cytolytic activity. In contrast, the population expressing a one-log higher level of CD56 (CD56\textsuperscript{bright}) is less cytotoxic and has a higher capacity to produce cytokines [1–8].

CD56\textsuperscript{dim} NK cells mainly produce cytokines when in contact with target cells [9, 10]. By contrast, CD56\textsuperscript{bright} NK cells are more likely to be activated by IL-15 produced and presented in trans by activated monocytes or DC or by IL-2 produced by CD4-positive
T cells during the onset of the adaptive immune response. CD56dim NK cells represent the major subpopulation in peripheral blood (>90%) but CD56bright NK cells may outnumber CD56dim NK cells because they are the predominant population in secondary lymphoid organs [7,11].

CD56dim and CD56bright NK cells display different combinations of cell surface receptors. CD56dim NK cells express high levels of the Fcγ receptor type III (CD16) and the chemokine receptors for IL-8 and fractalkine (CXCR1 and CX3CR1) to guide them into inflamed tissues [3,12]. By contrast, CD56bright NK cells use CCR5 for entry into inflamed tissues and CCR7 to home into secondary lymphoid organs, where they can be found in close vicinity of DC [6,13,14]. Both subpopulations express the IL-2 receptor β-chain and the common γ-chain that form heterodimers with low to intermediate affinity for IL-2 and IL-15 [15]. CD56bright NK cells also express low levels of the IL-2R α-chain (CD25) conferring specificity and high affinity for IL-2 [16] as well as c-kit [3,17–20], the type III tyrosine kinase receptor for SCF.

SCF is an essential growth factor for hematopoietic progenitor cells produced by stromal cells in bone marrow. Most hematopoietic lineages downregulate c-kit as they differentiate into more mature forms [21,22], but mast cells, eosinophils and CD56bright NK cells retain considerable levels of c-kit. During inflammation, fibroblasts and endothelial cells produce SCF [21,22]. Mature mast cells and eosinophils respond to SCF with chemotaxis [21,22], adhesion to fibronectin or endothelial cells [23–25] and degranulation [26]. The response of CD56bright NK cells to SCF has remained somewhat elusive. It has been shown that SCF decreases apoptosis in CD56bright NK cells stimulated with IL-2, enhancing proliferation and IFN-γ production in presence of IL-12 [17–20]. It is notable that these responses to SCF have been observed mainly in CD56bright NK cells cultured for several days. Even early signaling such as SCF-triggered phosphorylation of ERK through MAPK, the critical pathway to activate NK cells [27] has only been reported for NK-cell lines or for NK cells that had been stimulated with IL-2 [20]. Herein, we show that CD56bright NK cells in peripheral blood do not respond to SCF because they have downregulated c-kit. Furthermore, we provide data showing that the decrease of c-kit expression as well as the ensuing unresponsiveness to SCF is readily reversed by withdrawing the stimuli at their origin.

Results

Early signaling in primary CD56bright NK cells stimulated by IL-2, IL-7, IL-15, or SCF

Common γ-chain cytokines and SCF signal through MAPK-ERK, PI3K-Akt, and/or STAT-5 pathways. In NK cells, IL-2 and IL-15 have been shown to activate all three pathways [20,27–30]. IL-7 signaling has not been studied in detail. SCF induces ERK phosphorylation in hematopoietic progenitors [31], mast cells [22] as well as in c-kit positive endothelial cells [32] but apart from the observation that SCF enhances ERK phosphorylation in NK cells stimulated with IL-2 [20], little is known of its impact on NK cells ex vivo. We found that the response of peripheral blood CD56bright NK cells to IL-15 was very similar to the response described for CD56dim NK cells or for NK cell lines (Fig. 1). Within minutes after stimulation, 100 pM-2 nM of IL-15 induced maximal phosphorylation of STAT-5, ERK, and Akt while responses induced by concentrations lower than 100 pM were suboptimal. IL-2 also signaled through each of the three pathways, but on a molecular basis, appeared to be approximately one log less efficient than IL-15 for CD56bright NK cells and two logs less efficient for CD56dim NK cells. Furthermore, as reported for other lymphocytes, stimulation with IL-7 phosphorylated STAT-5, but not ERK [33,34]. IL-7 induced phosphorylation of Akt varied from negative to borderline in different experiments. CD56dim NK cells that do not express the IL-7 receptor [35] did not respond. We did not observe SCF-induced phosphorylation of ERK over a broad range of concentrations. Phosphorylation of Akt was undetectable at SCF-concentrations lower than 5 nM and marginal or negative at higher concentrations. Hence, CD56bright NK cells in peripheral blood respond to common γ-chain cytokines for which they express receptors but, in spite of expressing c-kit, do not respond to SCF.

CD56bright NK cells respond to SCF after separation from their cytokine milieu in vivo

CD56bright NK cells express c-kit and SCF has been shown to modulate their response to cytokines in culture [17–19]. The latter is hard to reconcile without SCF-induced phosphorylation of ERK. To test whether CD56bright NK cells had received signals in vivo interfering with SCF-induced phosphorylation of ERK, we monitored alterations of the response to SCF with time. We found that after 6–8 h of culture, CD56bright NK cells phosphorylated ERK and Akt (Fig. 2) but not STAT-5 (data not shown) in response to 10 nM of SCF. With time, sensitivity to SCF gradually increased. After 20 h, CD56bright NK cells phosphorylated ERK and Akt after stimulation by SCF-concentrations from 0.5–1 nM on (data not shown) while after 44 h, SCF concentrations as low as 2–5 pM induced half-maximal phosphorylation of ERK and Akt. By contrast, phosphorylation of STAT-5, ERK, and Akt after stimulation with the common γ-chain cytokines IL-7 and IL-15 was virtually identical (data not shown). Interestingly, CD56bright NK cells cultured in the presence of IL-7 became responsive to SCF in a similar manner but IL-15 blocked acquisition of responsiveness to SCF completely. Hence, signaling through c-kit is controlled by cytokines and while circulating in blood and lymph nodes, CD56bright NK cells may receive signals that negatively regulate SCF signaling through c-kit.

CD56bright NK cells that respond to SCF have upregulated c-kit

Receptor downregulation is a common mechanism to attenuate cellular responses to their specific ligands. Proinflammatory
signals such as IL-1β, TNF-α, IFN-γ, and GM-CSF downregulate c-kit on hematopoietic precursor cells and mast cells [21,22]. Hence, CD56<sub>bright</sub> NK cells ex vivo that express only low levels of c-kit may be unresponsive to SCF because they have downregulated c-kit in response to such stimuli in vivo. We have measured c-kit levels during the cultures depicted in Fig. 2 on cells that became responsive to SCF or not, and compared it with the levels of CD25 and CCR7 under the same conditions. We found that CD56<sub>bright</sub> NK cells cultured in the absence of cytokines expressed approximately 20 times more c-kit than CD56<sub>bright</sub> ex vivo. By contrast, the level of c-kit on CD56<sub>bright</sub> NK cells cultured in the presence of IL-2, IL-15, or SCF had changed only little. Hence, responses to SCF are associated with a high surface expression of c-kit.

It is notable that after 44 h of culture, we recovered fewer CD56<sub>bright</sub> NK cells from cultures without cytokines than from cultures containing IL-7. This was not unexpected because CD56<sub>bright</sub> NK cells undergo apoptosis after 2 days in culture without growth factors [18]. However, during the first 44 h of culture, we never observed differences in c-kit upregulation or responses to SCF of CD56<sub>bright</sub> NK cells cultured in medium without cytokines or in medium containing IL-7.

The change in receptor density on unstimulated CD56<sub>bright</sub> NK cells was highly specific for c-kit. CD25 was upregulated only after stimulation with IL-15, whereas CCR7 was downregulated only after stimulation with IL-2 or IL-15. Receptor expression was mainly regulated through transcription (Fig. 3, right panel) but the presence of the ligand-limited cell surface expression. CD56<sub>bright</sub> NK cells ex vivo and CD56<sub>bright</sub> NK cells stimulated with IL-2 or IL-15 transcribed c-kit at an almost identical level, which was significantly lower than that of unstimulated cells. Hence, transcription levels concurred accurately with the respective levels of c-kit at the cell surface. CD56<sub>bright</sub> NK cells stimulated with SCF transcribed c-kit at a similar level as unstimulated cells but expressed only low levels of c-kit, most likely because c-kit is rapidly internalized and degraded upon binding of its ligand [22,36]. The level of CD25-transcription increased sharply after stimulation with IL-2 or IL-15 but as has been reported for T cells [37], only cells
stimulated with IL-15 expressed high levels of CD25. Stimulation with IL-2 or IL-15 increased transcription of CCR7 during the first hours (not shown) but significantly decreased transcription thereafter, which led to the disappearance of CCR7 from the membrane during the second day of culture.

c-Kit downregulation by IL-15 and the ensuing SCF unresponsiveness is instantaneous but reversible

The fact that CD56<sup>bright</sup> NK cells cultured in the presence of IL-15 do not upregulate c-kit (Fig. 3) nor become responsive to SCF (Fig. 2) suggests that IL-15 is able to regulate responses to SCF. However, because CD56<sup>bright</sup> NK cells cultured in IL-15 enter into cell cycle and start to express markers that are considered to be features of CD56<sup>dim</sup> NK cells [7, 11], one could also argue that the IL-15 induced low levels of c-kit and unresponsiveness to SCF are simply hallmarks of cell division or of the start of a possible maturation into c-kit-negative CD56<sup>dim</sup> NK cells. To investigate this further, we cultured CD56<sup>bright</sup> NK cells for 44 h to induce maximal c-kit expression and stimulated with IL-7 or IL-15. CD56<sup>bright</sup> NK cells stimulated with IL-7 did not downregulate c-kit and remained fully responsive to SCF (Fig. 4A). By contrast, IL-15 swiftly downregulated c-kit to a level comparable...
Innate immunity

Figure 4. IL-15-induced downregulation of c-kit and SCF-responsiveness is instantaneous but reversible for at least 5 days. (A) c-kit expression and (B) SCF-induced ERK phosphorylation in CD56bright NK cells cultured for 44 h in the absence of cytokines followed by stimulation with IL-7 or IL-15 for 1–4 h. (C) c-Kit expression and (D) SCF-induced ERK phosphorylation in purified CD56bright NK cells cultured in IL-15 for 6 days; cultured in IL-15 for 5 days, washed and subsequently cultured in IL-7 for 1 day; or cultured in IL-15 for 4 days, washed and subsequently cultured in IL-7 for 2 days. Analysis of SCF-induced ERK phosphorylation was performed as in the previous figures. After withdrawal of IL-15, IL-7 was added to improve cell viability. Data are shown as mean ± SD of one sample from at least three independent experiments. Comparison of responses with the response of unstimulated cells (A, B) or with cells cultured in IL-15 for 6 days (C, D) was done as described in Fig. 3. (**p < 0.001, *p < 0.01, *p < 0.05).

Figure 5. SCF-signaling increases K562-induced CD107 expression in CD56bright NK cells that have upregulated c-kit. CD56bright NK cells ex vivo (left) or CD56bright/CD56dim cells (middle and right) previously cultured for 44 h under the conditions in Fig. 2 were stimulated overnight with SCF (5 nM) and assessed for expression of CD107 by FACS analysis. Each line represents an individual experiment.

SCF-signaling of CD56bright NK cells with upregulated c-kit increases their capacity to degranulate

Phosphorylation of ERK and Akt does not necessarily mean that SCF also activates signaling pathways downstream. To determine the impact of SCF on CD56bright NK cells, we incubated purified NK cells overnight with SCF and measured degranulation after stimulation with K562 target cells by the percentage of cells expressing CD107a with FACS gates on viable CD56bright or CD56dim NK cells that served as control. Very few NK cells (3.1 ± 2.4%, n = 20) degranulated without being stimulated with K562 (data not shown). Overnight incubation with SCF did not change the percentage of CD56bright NK cells ex vivo degranulating in response to K562 (20.1 ± 17.9 versus 22.0 ± 16.8, see left panel of Fig. 5 for five independent experiments). By contrast, SCF stimulation significantly increased the percentage of degranulating CD56bright NK cells that had upregulated c-kit during the preceding 44 h culture (21.2 ± 11.0 versus 35.9 ± 13.9 (<0.004), middle panel, n = 8), whereas the percentage of degranulating CD56dim NK cells cultured under the same conditions did not change (31.3 ± 17.1 versus 33.1 ± 15.7, right panel, n = 6). Hence, CD56bright NK cells respond to SCF by increasing their capacity to degranulate but only after they have upregulated c-kit.

CD56bright NK cells that have upregulated c-kit adhere to COS cells transfected with mSCF

SCF induces mast cell adhesion to fibronectin and endothelial cells [23–25]. To study whether CD56bright NK cells behaved similarly, we compared adherence to COS cells transfected with membrane bound SCF (mSCF) of CD56bright NK cells ex vivo to that of
CD56bright that had upregulated c-kit after 44 h of culture (Fig. 6). The number of CD56bright NK cells ex vivo adhering to mSCF-COS (1–2 CD56bright/COS) did not exceed background as defined by the number of CD56bright NK cells expressing high levels of c-kit adhering to mSCF-negative COS. By contrast, three times as many CD56bright NK cells adhered to mSCF-COS (5–6 CD56bright/COS) after upregulation of c-kit during 44 h of culture. Hence, besides increasing sensitivity to SCF signaling, upregulation of c-kit also increases adherence to cells expressing mSCF and it is possible that this contributes to the retention of CD56bright NK cells in inflammatory sites.

Discussion

NK cells have been named after their capacity to kill tumor cell lines without need for previous priming. To date, we begin to appreciate numerous other functions they may exert. This holds in particular for CD56bright NK cells, a subpopulation of NK cells that express a unique repertoire of cytokine, chemokine and adhesion receptors [3,12]. Many of these receptors are informative on CD56bright NK cells homing patterns and on how these cells may drive T cell polarization or assist in the control of viral infections. CD56bright NK cells express CCR7 and L-selectin that guides them to secondary lymphoid organs where they can be found in T cell area in close vicinity of DC [8, 14, 38, 39]. Furthermore, they express receptors for IL-12 and IL-15 [40,41] so that they are activated by DC that have sensed danger signals as well as receptors for IL-2 and IL-4 to interact with a concomitant T-cell response [2, 6, 16]. Once activated, they leave the secondary lymphoid organs and are guided by CCR5 to tissues where they contribute to the inflammatory response [8,42–44].

CD56bright NK cells also express c-kit [3,17–20]. Mast cell precursors in blood use c-kit to adhere to endothelial cells that express mSCF under inflammatory conditions. In addition, c-kit-mediated signaling contributes to their maturation and survival after entry into inflamed tissues [21, 22, 24, 25]. It is conceivable that CD56bright NK cells follow similar routes but after its discovery more than 20 years ago [17], the interest in c-kit on NK cells has somewhat faded not only because the effects of SCF on CD56bright NK cells appeared to be only minor [17–20] but also because CD56bright NK cells are considered by many as a sort of precursor of CD56dim NK cells [7,11,45,46], which would demote c-kit to a receptor on its way out. Recently, we have reported that after hematopoietic stem cell transplantation, CD56bright NK cells in transplanted patients and in control individuals are modulated by cytokines [47]. In this report, we show underlying mechanisms and functional consequences. We believe that these findings allow speculating on features of CD56bright NK cells complementary to those inferred from their repertoire of chemokine and adhesion receptors.

We found that CD56bright NK cells in peripheral blood express only low levels of c-kit and do not respond to SCF. The latter

![Figure 6. CD56bright NK cells with upregulated c-kit adhere to COS cells transfected with mSCF. Adherence of either (A) purified CD56bright NK cells ex vivo or (B) purified CD56bright NK cells expressing high levels of c-kit after 44 h in culture to mSCF-GFP-expressing COS cells was examined by phase-contrast microscopy (magnification 63). The inserts show immunofluorescence in the same field, depicting the presence mSCF-GFP in the transfected cells. (C) The mean number of CD56bright cells adhered to COS cells counted per field is shown, which corresponds approximately to the number of CD56bright adhered to 1.3 COS cells. Data are shown as mean ± SD of at least 13 fields from each of the three independent experiments. Adherence of purified CD56bright NK cells expressing high levels of c-kit to COS-mSCF (right bar) was compared with adherence of CD56bright NK cells ex vivo to COS-mSCF (left bar) or with adherence of CD56bright NK cells expressing high levels of c-kit to untransfected COS cells (middle bar) as described in the previous figures (***p < 0.01).](image-url)
makes sense because responses to SCF present in blood should be avoided. We also show that downregulation of c-kit renders CD56^{bright} NK cells nonresponsive to SCF. Although it took 6–8 h of culture to upregulate c-kit to a level that CD56^{bright} NK cells responded to SCF in vitro, this process could well be faster after cells have extravasated and are stimulated by mSCF on cells simultaneously presenting ligands for other receptors. Indeed, thresholds are likely to be different in vivo when CD56^{bright} NK cells receive other signals that might be accessory. Hence, once extravasated, upregulation of c-kit may not only render CD56^{bright} NK cells sensitive to SCF-induced survival signals but also firmly anchor them to cells expressing mSCF.

We used IL-15 to show that CD56^{bright} NK cells shut down responsiveness to SCF immediately after being stimulated. We would have obtained similar results with IL-2 but we have no indication that one of these cytokines is the cause of the downregulation of c-kit on peripheral blood CD56^{bright} NK cells in vivo. SCF also downregulates c-kit (Fig. 3) but its concentration in plasma 242.1 ± 23.0 pg/mL [48] is too low by 2 logs to have an impact (data not shown). Downregulation may have occurred in LN on encounter with DC presenting IL-15 or in response to other cytokines with similar effects. We did observe that the upregulation of c-kit on CD56^{bright} NK cells and its ensuing increase of responsiveness to SCF in cultures of peripheral blood mononuclear cells were less pronounced than in cultures of purified NK cells. Although it is tempting to speculate that the membrane bound IL-15 on monocytes present in the same culture prevented much of the upregulation, we have not been able to pinpoint the cause exactly and many other signals may also contribute.

Our crucial finding is the fact that peripheral blood CD56^{bright} NK cells do not respond to SCF does not mean that will not do so later on. Furthermore, we show that commitment to a c-kit-negative NK cell is not readily obtained by stimulating CD56^{bright} NK cells with IL-15 because even after one week, withdrawal almost immediately restores c-kit to a level that is one log higher than found on CD56^{bright} NK cells in peripheral blood. We also tested complexes of IL-15 and its soluble receptor a-chain that signals human NK cells in a similar way as IL-15 presented in trans [30,46], but did not find indications that CD56^{bright} NK cells would become more committed to a c-kit-negative cell than after stimulation with IL-15 alone (data not shown).

Collectively, our data suggest that peripheral blood CD56^{bright} NK cells are a distinct subpopulation of NK cells that migrates to inflammatory sites and becomes effector cells after stimulation by antigen-presenting cells [8, 14, 42, 43], endothelial cells, or platelets [44, 49]. Although during this process, CD56^{bright} NK cells may acquire features of CD56^{dim} NK cells [7, 11], we do not believe that this is a major pathway for the generation of CD56^{dim} and that it may be time to fine-tune the current paradigm of peripheral blood CD56^{bright} NK cells being precursors of CD56^{dim} NK cells.

### Materials and methods

#### NK cell isolation and culture

Purified NK cells or purified CD56^{bright} NK cells were obtained from buffy coats from healthy blood donors by standard gradient Ficoll density centrifugation and MACS using the human NK Cell Isolation Kit or the CD56^+ CD16^+ human NK Cell Isolation Kit, respectively (Miltenyi Biotec, Bergisch Gladbach, Germany), according to manufacturer’s instructions. Purities were always >90 for NK cells and > 75% for CD56^{bright} NK cells. Blood donors (age 45 ± 16, 55% male, 45% female) from our blood transfusion center had given consent and the study had been approved by our institutional ethical committee. Purified NK cells were cultured in AIMV medium (Life Technology, Zug, Switzerland) containing 2% Hepes and incubated at 37 °C in the presence or absence of IL-2, IL-7, IL-15, or SCF (Miltenyi Biotec) for the indicated time. Because CD56-upregulation by CD56^{dim} NK cells from the third day of culture would render discrimination of CD56^{bright} from CD56^{dim} by FACS less precise, cultures longer than 44 h were always performed with purified CD56^{bright} NK cells.

#### FACS analysis and Phosflow staining

After staining with fluorochrome-conjugated antibodies, FACS analysis was performed using a Gallios (Beckton Coulter, Nyon, Switzerland) and FlowJo software. Sytox Blue (Invitrogen, Basel, Switzerland) gating was used to exclude dead cells. The following antibodies were used: PE labeled anti-CD56 (AF12–7H3) from Miltenyi, AlexaFluor488 labeled anti-pERK1/2 (20A), AlexaFluor647 labeled anti-pSTAT5 (47/Stat5pY694), and AlexaFluor647 labeled anti-pAkt (M89–61) were from BD biosciences, Allschwil, Switzerland, allophycocyanin labeled anti-CD117 (104D2), PE labeled anti-CD25 (BC96), BrilliantViolet421 labeled anti-CD56 (HCD56) from Biolegend, Luzern, Switzerland and FITC labeled anti-CCR7 (150503) from R&D Systems, Abingdon, UK. For Phosflow-staining, purified NK cells or purified CD56^{bright} NK cells were first incubated in medium with cytokines (see above) for 0–20 min at 37 °C. Equal volumes of pre-warmed BD Cytofix fixation buffer (BD Biosciences) was then added to the cell suspension and fixation was carried out at 37 °C for 15 min. Cells were then washed with PBS containing 0.1% BSA, incubated at 4 °C for 30 min in 200 μL of ice-cold BD Phosflow Perm Buffer III (BD Biosciences) and washed before staining with antibodies at room temperature. Different phosphorylation states were expressed as geometric mean fluorescence intensity ratios calculated by dividing the MFI of the stimulated sample by the MFI of the corresponding unstimulated sample.

NK cell degranulation (CD107a expression) was measured as published by Alter et al. [50] by stimulating NK cells that had been cultured for 20 h ± SCF for 4 hours at 37 °C with K562 cells.
at an E:T ratio of 1:1 in the presence of an APC labeled anti-CD107a antibody (H4A3 BD Biosciences). After 1 hour of culture 0.5 μL/mL of Golgistop (BD Biosciences) was added. Thereafter, NK cells were washed and stained with BrilliantViolet421 labeled anti-CD56 for 30 min at 4°C for FACS analysis with gates on viable cells using Sytox Blue.

RNA isolation and RT-PCR

RNA isolation from cells cultured under the conditions described above was performed with the RNeasy Mini kit (Qiagen, Magden, Switzerland), according to the manufacturer’s instructions. Complementary strand synthesis was performed with 0.1 μg of isolated total RNA using 0.5 μg random primers, 3mM MgCl₂, 0.5mM dNTP, 20 U RNasin Ribonuclease inhibitor and 160U of ImProm II reverse transcriptase (Promega, Dübendorf, Switzerland). Reversed transcription was carried out at 25°C for 5 min, 42°C for 60 min followed by 70°C for 15 min. PCR was performed using QuantiTect SYBR Green PCR Kit and QuantiTect Primer Assays for c-kit, CCR7, CD25, and Tata Box-binding protein (TBP, Qiagen) at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The 2−ΔΔCT method was used to analyze real-time PCR data. The expression of each gene was normalized against the housekeeping gene TBP.

NK adhesion to mSCF-GFP transfected COS-7 cells

Twenty-four hours after seeding on glass coverslips, COS-7 cells (American Type Culture Collection) were transiently transfected using Xtrem-9 (Roche, Indianapolis, IN, USA) with constructs containing the sequence of the membrane bound form of human SCF (mSCF) coupled to the sequence of Green Fluorescence Protein (mSCF-GFP) [51]. CD56bright NK cells were added (900 cells/mm²) and fixed after 1 h for 15 min in 4% paraformaldehyde/PBS. After washing, phase contrast and GFP fluorescence images (filter set XF116; Omega, Brattleboro, VT) were taken with a PlanNeofluar 63× NA 1.4 oil immersion objective (Zeiss, Zurich, Switzerland) on a Zeiss-Axiovert100TV microscope (Zeiss) equipped with a 10-bit CCD camera (Hamamatsu Photonics, Tokyo, Japan), controlled by the Openlab software (PerkinElmer, Waltham, MA).

Statistics

We used GraphPad Prism software for statistical analysis computing statistical significance between groups by one-way ANOVA followed by a Tukey test to compare means of several experiments or t-tests when appropriate. p-values < 0.05 were considered significant and are shown as *** when p < 0.001, ** when p < 0.01 and * when p < 0.05.

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