Production of large numbers of plasmacytoid dendritic cells with functional activities from CD34+ hematopoietic progenitor cells: Use of interleukin-3

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(Received 10 August 2011; revised 20 December 2011; accepted 3 January 2012)

Plasmacytoid dendritic cells (pDC), a subset of dendritic cells characterized by a rapid and massive type-I interferon secretion through the Toll-like receptor pathway in response to viral infection, play important roles in the pathogenesis of several diseases, such as chronic viral infections (e.g., hepatitis C virus, human immunodeficiency virus), autoimmunity (e.g., psoriasis, systemic lupus erythematosus), and cancer. As pDC represent a rare cell type in the peripheral blood, the goal of this study was to develop a new method to efficiently generate large numbers of cells from a limited number of CD34+ cord blood progenitors to provide a tool to resolve important questions about how pDC mediate tolerance, autoimmunity, and cancer. Human CD34+ hematopoietic progenitor cells isolated from cord blood were cultured with a combination of Flt3-ligand (Flt3L), thrombopoietin (TPO), and one of the following cytokines: interleukin (IL)-3, interferon-β, or prostaglandin E2. Cells obtained in the different culture conditions were analyzed for their phenotype and functional characteristics. The addition of IL-3 cooperates with Flt3L and TPO in the induction of pDC from CD34+ hematopoietic progenitor cells. Indeed, Flt3L/TPO alone or supplemented with prostaglandin E2 or interferon-β produced smaller amounts of pDC from hematopoietic progenitor cells. In addition, pDC generated in Flt3L/TPO/IL-3 cultures exhibited morphological, immunohistochemical, and functional features of peripheral blood pDC. We showed that IL-3, in association with Flt3L and TPO, provides an advantageous tool for large-scale generation of pDC. This culture condition generated, starting from 2 × 10^5 CD34+ cells, up to 2.6 × 10^6 pDC presenting features of blood pDC. © 2012 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.
regulation and activities, especially in tolerance, autoimmunity, and cancer. The obtention of pDC in large numbers has proven to be difficult, limiting progress in the understanding of their functions. Here, we describe a simple method for the large-scale generation of pDC exhibiting morphological, immunohistochemical, and functional features of pDC present in the peripheral blood from a limited number of CD34⁺ cord blood progenitors.

Several cytokines, such as IL-3, prostaglandin E2 (PGE₂), and IFN-β have been shown to be implicated in pDC development, proliferation, and/or survival. IL-3 induces the proliferation of pDC, inhibits their apoptosis [14,15], and has a proliferative effect on CD34⁺ progenitor cells [16–18]. CD34⁺ cells cultured with mesenchymal stem cells or their conditioned medium have been shown to increase the number of cells generated and the percentage of pDC in culture through PGE₂ production [19]. Buelens et al. showed that monocytes cultured in IL-3 and IFN-β give rise to a population of DC showing several characteristics of pDC. Those cells express high levels of CD123 and secreted high levels of IL-6 and tumor necrosis factor–α [20]. To determine the best method to generate high amounts of pDC in vitro, we added one of those cytokines (i.e., IL-3, IFN-β, or PGE₂) to Flt3 ligand (Flt3L)/thrombopoietin (TPO) CD34⁺ hematopoietic progenitor cells (HPC) culture as previous studies showed that TPO acts in synergy with Flt3L for the differentiation and the expansion of HPC [21].

We showed that the conditions Flt3L/TPO, Flt3L/TPO/PGE₂, and Flt3L/TPO/IFN-β induced generation of low numbers of pDC. In contrast, TPO, Flt3L, and IL-3 synergistically induce generation of >2.6 × 10⁶ pDC presenting characteristics of peripheral blood pDC from a small initial number of precursor cells (2 × 10⁵ HPC) after 21 days in culture. These findings suggest that, unlike IFN-β and PGE₂, IL-3 might represent a key factor in controlling pDC development.

Materials and methods

Isolation of pDC from peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from leukocyte-enriched Buffy-coats by centrifugation on Ficoll-Hypaque (Lymphoprep, Axis-Shield, Oslo, Norway). After washing PBMC at low centrifugation speed to discard a maximum of platelets, pDC were sorted by using a negative cell sorting kit following manufacturer’s instructions (Human Plasmacytoid DC Enrichment kit; StemCell Technologies, Vancouver, Canada).

Generation of pDC from CD34⁺ HPC

CD34⁺ HPC were isolated from human umbilical cord blood obtained during normal full-term deliveries. This study was approved by the Ethics Committee of the University Hospital of Liege and informed consent was obtained from donors. CD34⁺ HPC were recovered by Ficoll-Hypaque density-gradient centrifugation (Lymphoprep; Axis-Shield). CD34⁺ cells were isolated from other mononucleated cells using the MACS Direct CD34 Progenitor Cell Isolation Kit (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and MiniMacs separation columns (Miltenyi Biotec), according to manufacturer’s protocol. CD34⁺ cells were cultured in 24-well plates (Nunc, Roskilde, Denmark) at 2 × 10⁵ cells/mL in RPMI medium supplemented with 10% fetal calf serum, 50 μM β-mercaptoethanol and 1% penicillin-streptomycin, sodium pyruvate, and nonessential amino acid and (all purchased from Invitrogen, Merelbeke, Belgium). To obtain pDC differentiation, TPO (50 ng/mL), Flt3L (100 ng/mL), and one of the following recombinant human cytokines: IL-3 (20 ng/mL), IFN-β (1000 U/mL), or PGE₂ (15 ng/mL), were added to the medium. All cytokines were purchased from Peprotech (Rocky Hill, NJ, USA) except for PGE₂ (Cayman Chemicals, Ann Arbor, MI, USA). Cell cultures were refreshed every 3 days with RPMI medium containing designated cytokines. pDC were isolated by using the Human Plasmacytoid DC Enrichment kit (StemCell Technologies). In some experiments, pDC were stimulated during 24 hours with a CpG oligodeoxynucleotide (ODN) at 12 μg/mL (CpG ODN 2216; 5’-ggGGGACGATGCCTGCCCCGGGGG-3’; Eurogentec, Seraing, Belgium).

Flow cytometry analysis

Flow cytometry studies were performed by using procedures published previously [22] with the following antibodies: CD123-FITC (clone AC145; Miltenyi Biotec), CD11c-allophycocyanin (clone Q6B-ly6; BD Pharmingen, Franklin Lakes, NJ, USA), BDCA-4-phycocerythrin (PE) (clone AD5-17F6; Miltenyi Biotec), CD40-PE (clone 5C3; BD Pharmingen), CD86-PE (clone HB15e; BD Pharmingen), CCR7-PE (clone 150503, R&D Systems, Minneapolis, MN, USA), and HLA-DR-PE (clone AB3; Dako, Glostrup, Denmark). Fluorescence intensity and positive cell percentages were measured on a FACScanto (Becton Dickinson, NJ, USA) and data were analyzed using FACSDiva software V 6.1.2 (Becton Dickinson) and FlowJo software (TreeStar, Ashland, OR, USA).

Reverse transcription polymerase chain reaction analysis

One microgram total RNA extracted from cell cultures (RNeasy mini kit; Qiagen, Valencia, CA, USA) was reverse-transcribed using Superscript II reverse transcriptase (Invitrogen) according to manufacturer’s instructions. Reverse transcription polymerase chain reaction (RT-PCR) were performed using the following primer sequences: TLR9 F: ACTGGAGGTGGCCCCGGGGA; TLR9 R: CAGGGGTTGGGACGTGG; HPRT F: GTTGAGATAGCCAGACTTGGTT; and HPRT R: CAGATTTCCCCTAACTGACGTGA (Eurogentec). The housekeeping gene HPRT was used as an internal control.

Cytokine production assays

Culture supernatants collected from pDC cultures were assayed for IFN-α levels by using an enzyme-linked immunosorbent assay kit according to manufacturer’s instructions (PBL Interferon Source, Piscataway, NJ, USA).

pDC chemotaxis assay

pDC migration was evaluated using a chemotaxis microchamber technique (48-well Boyden microchamber; Neuroprobe, Cabin John, MD, USA) [23]. Human recombinant chemerin (10 pM, 100 pM, 10 nM, or 100 nM; R&D Systems) was added to the lower wells of the chamber. A nonconditioned medium was used as
control for random migration. Conditioned medium of human fibroblasts was used as positive control. A polyvinylpyrrolidone-free polycarbonate membrane 5-μm gelatin-coated pore filter (Poretics Corp., Livermore, CA, USA) was placed in the microchamber. After cell sorting, 55 μL pDC suspension (2 × 10^6 cells/mL) was applied into the upper wells of the chamber. The chamber was incubated for 5 hours at 37°C. Cells having migrated to the underside of the filter were fixed and stained with Diff Quick Stain set (Baxter Diagnostics AG, Düdingen, Switzerland). The upper side of the filter was scraped to remove residual nonmigrating cells. One random field was counted per well using an eyepiece with a calibrated grid to evaluate the number of fully migrated cells.

**Mixed lymphocyte reaction assay**

The stimulator population consisted of pDC sorted from peripheral blood or Flt3L/TPO/IL-3 culture. Those cells were irradiated at 5000 rads and placed in RPMI 5% human pooled AB serum. Varying numbers of stimulator cells (312,400,000 cells per well) were added to round-bottomed 96-well Nunclon plates containing 2 × 10^5 allogeneic PBMC per well. A proliferative response was measured after 5 days of culture by adding 1 μCi ^3H-thymidine to each well. Cells were harvested 18 hours later using an automated sample harvester (Packard, Canberra, Tilburg, The Netherlands) and counted using a liquid scintillation counter (Top Count, Packard). DCs obtained by culturing adherent fraction of PBMC with Q9 IL-4 and granulocyte-macrophage colony-stimulating factor, as described previously [22,24], were used as positive control.

**Morphology of pDC**

Cytopsins of pDC sorted from peripheral blood or Flt3L/TPO/IL-3 cultures were prepared by spinning (200 rpm, 3 minutes) 1 × 10^5 cells onto methanol-treated slides. May–Grünwald Giemsa staining was performed using standard procedures and slides were examined using a FSX100 microscope (Olympus, Aartselaar, Belgium).

**Statistical analysis**

Statistical evaluation of the results was performed using unpaired Student’s t test. Comparisons of means were studied by analysis of variance (ANOVA), followed by a Newman–Keuls multiple comparison test (one-way ANOVA) or a Bonferroni post-test (two-way ANOVA). ANOVA tests were performed on log-transformed data. Differences were considered as statistically significant when p < 0.05. Statistical tests were performed using the GraphPad Prism 5 software (Graph-Pad Software, La Jolla, CA, USA).

**Results**

**IL-3 induces massive expansion of HPC and generation of large numbers of pDC from CD34+ HPC cultivated in the presence of Flt3L and TPO**

Previous works showed that TPO cooperates with Flt3L in the generation of pDC from CD34+ HPC [21]. In this study, we tested the combinations of Flt3L (100 ng/mL) and TPO (50 ng/mL) with other growth factors that have been shown to be implicated in pDC development, proliferation, and/or survival, such as IL-3 (20 ng/mL), IFN-β (1000 U/mL), and PGE2 (15 ng/mL) [14,16,17,19,25].

In six different experiments, 2 × 10^5 CD34+ HPC were incubated in media containing Flt3L/TPO alone or with IL-3, IFN-β, or PGE2 for 28 days. Total number of cells was counted and analyzed at days 14, 21, and 28. Data shown represent mean ± standard deviation of six independent experiments using different donors.

![Figure 1](image-url)  
*Figure 1. IL-3 in combination with Flt3L and TPO promotes the proliferation of CD34+ HPC. CD34+ HPC were cultured with Flt3L and TPO alone or with IL-3, PGE2, or IFN-β for 28 days. Total number of cells were counted and analyzed at days 14, 21, and 28. Data shown represent mean ± standard deviation of six independent experiments using different donors.*

In three independent experiments, we also analyzed the percentages of hematopoietic cell types present in our culture conditions. Cells were examined for their expression of markers related to pDC (BDCA-4+/CD123+/CD11c+), mDC (CD11b+/CD14+), T cells (CD3+), natural killer cells (CD3−/CD16+), B cells (CD19+), granulocytes (CD15+), eosinophils (CD16+), and monocytes (CD14+) by flow cytometry. We showed that mDC, T cells, and granulocytes were the main cell types produced in the different culture conditions (Fig. 2). By using an anti-CD34 antibody we also showed that only a small number of CD34+ HPC remained in the culture medium after 21 days of culture, as in Flt3L/TPO,
Figure 2. Various hematopoietic cell types are produced in Flt3L/TPO culture alone or supplemented with IL-3, PGE2, or IFN-β. Percentages of pDC (BDCA-4+/CD123+/CD11c+) (A), mDC (CD1a+/CD11c+) (B), T cells (CD3+) (C), B cells (CD19+) (D), natural killer cells (CD3-CD16+) (E), granulocytes (CD3-CD11a+/CD14+) and CD3-CD11a-/CD14-) (F), and monocytes (CD3-CD11a-/CD14+) (G) in cultures, at days 14 (light gray), 21 (dark gray), and 28 (black), were determined by flow cytometry using their corresponding phenotypes. Data are from three different experiments using different donors and mean values are shown as percentages of positive cells ± standard deviation.
Flt3L/TPO/IL-3, Flt3L/TPO/PGE2, and Flt3L/TPO/IFN-β conditions, only up to 6.2%, 0.8%, 2%, and 1.3% of cells were CD34+ HPC, respectively (data not shown). At day 14, the maximum yield of pDC was observed using IL-3 in the culture medium. With this condition, 4% ± 1.9% of cells showed a phenotype of fully differentiated pDC (BDCA-4+/CD123+/CD11c+). The range of increase compared with pDC obtained at day 14 in other culture conditions was from >170% to 500%. After long-term cultures (28 days), the percentage of pDC increased and remained the highest in Flt3L/TPO/IL-3 culture (Fig. 2A).

Next, we determined the number of immature pDC based on their percentages in culture and showed that the combination of Flt3L, TPO, and IL-3 induced an enhancement in pDC generation. At 14, 21, and 28 days of culture, the number of pDC was higher in Flt3L/TPO/IL-3 condition compared with the other culture conditions (Fig. 3). The highest number of pDC was detected at day 21 in Flt3L/TPO/IL-3 culture (2.6 × 10^6 ± 1.2 × 10^6) and the number of pDC was significantly higher in Flt3L/TPO/IL-3 compared with Flt3L/TPO (p < 0.05), Flt3L/TPO/PGE2 (p < 0.05), and Flt3L/TPO/IFN-β (p < 0.01) cultures. These results are concordant with apoptotic tests showing that the Flt3L/TPO/IL-3 condition presents the highest percentages of living cells in culture. Proliferation tests also showed that at day 14, 21, and 28 of culture, cell proliferation was higher in Flt3L/TPO/IL-3 condition than in the other conditions tested. Flt3L/TPO/PGE2 condition was characterized by low percentages of proliferating cells. In addition, IFN-β strongly blocked pDC generation from HPC in Flt3L/TPO culture, as this culture condition was characterized by low percentages of proliferating cells and high numbers of apoptotic cells (data not shown).

We showed that Flt3L/TPO/IL-3 condition was responsible for the generation of the highest amount of pDC in vitro. We therefore decided to study more extensively cells obtained only with this condition. In addition, as pDC numbers were the highest at day 21 in Flt3L/TPO/IL-3 culture, subsequent studies were performed on pDC obtained after 21 days of culture.

**pDC derived from HPC in Flt3L/TPO/IL-3 culture differentiate into mature pDC after activation by CpG ODN**

We performed a phenotypic analysis on pDC sorted from Flt3L/TPO/IL-3 culture at day 21. After pDC isolation, we showed that a majority of cells were CD11c−. Only 2% of cells were CD11c+, BDCA-4+/CD123+ pDC represented 25.7% of CD11c− cells. Interestingly, 11.2% of CD11c− cells expressed the pDC markers BDCA-4+ and CD123+ (Fig. 4A). Sorted-pDC were then cultured 24 hours with IL-3 to assure their survival and with CpG ODN (a pDC stimulus recognized by their TLR9) to induce their maturation. Phenotype analysis of sorted cells cultured in medium complemented with IL-3 alone showed that the number of CD11c− and CD11c+ cells expressing the markers BDCA-4 and CD123 is increased in the presence of IL-3 from 25.7% to 53.4% and from 11.2% to 33.4%, respectively (Fig. 4B). Sorted cells cultured for 24 hours with IL-3 and CpG ODN showed a higher increase of BDCA-4+/CD123+/CD11c− (from 25.7% to 91.5%) and BDCA-4+/CD123+/CD11c+ (from 11.2% to 77.5%) cells compared with sorted cells cultured with IL-3 alone (Fig. 4C). These results demonstrated that BDCA-4+/CD123+ CD11c− cells are immature pDC that differentiate into mature pDC expressing CD11c when cultured with IL-3, with or without CpG ODN. However, the combination of IL-3 and CpG ODN induced a higher number of mature pDC than IL-3 alone.

We were also interested in determining whether nonsorted pDC exposed to Flt3L, TPO, and IL-3 were capable of maturation with CpG ODN. Nonsorted pDC generated in Flt3L/TPO/IL-3 condition were assessed for their expression of maturation markers (CD40, CD83, CD86, HLA-DR, CCR7, and CD11c) in the presence or absence of CpG ODN. After 24 hours of activation by CpG ODN, we showed that pDC stimulated with CpG ODN were matured. The fluorescence intensity of all maturation markers was increased in stimulated-pDC compared to non-stimulated pDC (Fig. 5).

**pDC generated in Flt3L/TPO/IL-3 cultures display characteristics of peripheral blood pDC**

In order to determine whether pDC generated in Flt3L/TPO/IL-3 culture display characteristics of peripheral blood pDC, we sorted pDC from Flt3L/TPO/IL-3 cultures and from peripheral blood by magnetic bead isolation.

Sorted-pDC from Flt3L/TPO/IL-3 cultures (Fig. 6Aa) exhibited typical peripheral blood pDC plasma cell...
morphology (Fig. 6Ab) with a high nucleus–cytoplasm ratio on a May–Grünwald Giemsa–stained cytopsin.

By using a mixed lymphocyte reaction, we showed that pDC generated in Flt3L/TPO/IL-3 culture as well as peripheral blood pDC have the capacity to provide accessory signals for an efficient proliferation of allogeneic T lymphocytes (Fig. 6B). pDC generated in culture or sorted from peripheral blood did not differ in their ability to stimulate an allogeneic response when the stimulator/responder ratio was the lowest (1:40 and 1:20), but the response was significantly more important for the population of pDC generated in culture when the stimulator/responder ratio was higher (1:10 and 1:5) ($p < 0.05$). In addition, as it has already been shown [21,26], the stimulatory capacity of pDC was significantly less important ($p < 0.01$) than that of the mDC population used as control in the assay (Fig. 6B).

pDC function is associated with the expression of TLR different from that expressed by mDC [27]. We confirmed by quantitative real-time PCR that, unlike mDC, peripheral blood pDC and pDC produced in our culture system, express high levels of TLR9 messenger RNA (Fig. 6C).
This messenger RNA expression was correlated to the TLR9 protein level detected in pDC by intracellular staining (Fig. 6D).

We also showed that in pDC sorted from the Flt3L/TPO/IL-3 culture, TLR9 stimulation by CpG ODN induces the secretion of high amounts of IFN-α (Fig. 6E) compared with unstimulated pDC.

As pDC migrate in response to chemerin due to their expression of CMKLR1 [28–30], we investigated the ability of pDC generated in Flt3L/TPO/IL-3 cultures to migrate in the presence of human recombinant chemerin by using a Boyden chamber assay. A significantly increased migration of pDC was observed in the presence of human fibroblasts media ($p < 0.001$) compared with a nonconditioned medium as control. We also showed that different concentrations of chemerin (100 μM, 10 nM, and 100 nM) induced a significantly increased migration of pDC, with a peak observed at 10 nM, compared with the nonconditioned medium (Fig. 6F).

These results demonstrated that pDC generated in vitro have characteristics and functional properties similar to those of peripheral blood pDC.

**Discussion**

Because of their unique ability to secrete IFN-α and their central roles in innate and adaptive immunity, pDC have attracted growing interest for several years. pDC investigation is also of potential interest because of their implication in the pathogenesis of several diseases in which they often present a pathogenic/tolerogenic role mainly related to either the increase or the reduction of their function [13]. Sustained overproduction of type-I IFN by pDC in response to host-derived self-nucleic acid is associated with the development of autoimmunity in systemic lupus erythematosus [31] and Q11 psoriasis [32]. On the other hand, pDC have also been shown to present a limited responsiveness to certain viruses (e.g., hepatitis C virus and human immunodeficiency virus) Q12 [33,34]. In addition, in the microenvironment of certain tumors [35–37], pDC are often responsible for the development of an immunosuppressive/tolerogenic response [38].

Although the function and clinical manipulation of pDC have become topics of great interest, progress in the understanding of pDC is limited by their low percentage in peripheral blood and the difficulty producing large quantities of these cells in culture. Several groups have already
induced the differentiation of pDC in vitro from CD34⁺ HPC [14,19,21,39,40]. However, these approaches generated relatively small amounts of pDC. Here, we describe an original procedure using a combination of Flt3L, TPO, and IL-3 for large-scale generation of pDC exhibiting morphological, immunohistochemical, and functional features of pDC present in the peripheral blood from a limited number of CD34⁺ HPC.
Originally, pDC were thought to be in the lymphoid lineage. Interestingly, recent studies indicate that these cells have diverse origins and can develop from lymphoid or myeloid precursors [41,42]. Even if the origin and affiliation of pDC is controversial, partly because pDC show features of lymphocytes and DC, clear evidences indicate that Flt3L and TPO might represent major regulators of pDC development. First, previous reports showed that Flt3L is responsible for development of pDC in vitro [21,39,43,44] and in vivo [45]. Interestingly, mice and human subjects injected with Flt3L have an increase in pDC number [46–48]. Second, TPO has been shown to support the proliferation and long-term expansion of HPC and to induce their differentiation into pDC in synergy with Flt3L [21,49,50]. As IL-3 was reported to induce the proliferation of pDC, to inhibit their apoptosis [14,15] and to have a proliferative effect on CD34 cells [51], it must be emphasized that CD11c by Buelens et al. [20] and Huang et al. [25].

At day 21, the combination of Flt3L, TPO, and IL-3 was found to support the differentiation and proliferation of human pDC from CD34 progenitor cells, of a large number of cells with phenotypic characteristics of pDC precursors, as a fraction of those cells differentiated into CD11c+CD123+ pDC sorted from cultures might be pDC precursors, as a fraction of those cells differentiated into CD11c+CD123+ pDC sorted from cultures might have diverse origins and can develop from lymphoid or myeloid precursors [41,42]. Even if the origin and affiliation of pDC is controversial, partly because pDC show features of lymphocytes and DC, clear evidences indicate that Flt3L and TPO might represent major regulators of pDC development. First, previous reports showed that Flt3L is responsible for development of pDC in vitro [21,39,43,44] and in vivo [45]. Interestingly, mice and human subjects injected with Flt3L have an increase in pDC number [46–48]. Second, TPO has been shown to support the proliferation and long-term expansion of HPC and to induce their differentiation into pDC in synergy with Flt3L [21,49,50]. As IL-3 was reported to induce the proliferation of pDC, to inhibit their apoptosis [14,15] and to have a proliferative effect on CD34 cells [51], it must be emphasized that CD11c by Buelens et al. [20] and Huang et al. [25].

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CD123+ cells was 1.7 fold more important than the number of BDCA4+/CD123+ cells.

Achievement of generation of high amounts of pDC in vitro opens up an exciting possibilities for the initiation of functional studies allowing for better comprehension of pDC regulation and function in homeostatic and pathological conditions, and also for the development of therapeutic approaches targeting human pDC.

Funding disclosure
This work was supported by a grant from the FNRS (Bourse Télève) and the GIGA imaging and flow cytometry platform.

Conflict of interest disclosure
No financial interest/relationships with financial interest relating to the topic of this article have been declared.

References


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<tr>
<td>Q1</td>
<td>Please provide degree initials for corresponding author.</td>
</tr>
<tr>
<td>Q2</td>
<td>SLE expanded to systemic lupus erythematosus Please correct if necessary.</td>
</tr>
<tr>
<td>Q3</td>
<td>Many of the Greek letters in your text had to be re-input. Please check them carefully as you review your proofs.</td>
</tr>
<tr>
<td>Q4</td>
<td>NK expanded to natural killer Please correct if necessary.</td>
</tr>
<tr>
<td>Q5</td>
<td>In line “In some experiments,……….,” please check whether the unit is correct or not after 2.</td>
</tr>
<tr>
<td>Q6</td>
<td>APC has been expanded to « allophycocyanin” Please correct if necessary.</td>
</tr>
<tr>
<td>Q7</td>
<td>PE has been defined as phycoerythrin Please correct if necessary.</td>
</tr>
<tr>
<td>Q8</td>
<td>gelatin-coated” moved here from after parentheses. Please correct if necessary.</td>
</tr>
<tr>
<td>Q9</td>
<td>GM-CSF has been expanded to granulocyte-macrophage colony-stimulating factor Please correct if necessary.</td>
</tr>
<tr>
<td>Q10</td>
<td>NK expanded to « natural killer » please correct if necessary.</td>
</tr>
<tr>
<td>Q11</td>
<td>SLE expanded to systemic lupus erythematosus Please correct if necessary.</td>
</tr>
<tr>
<td>Q12</td>
<td>HCV and HIV expanded to hepatitis C virus and human immunodeficiency virus” Please correct if necessary.</td>
</tr>
<tr>
<td>Q13</td>
<td>Please provide reference number for Chen et al.</td>
</tr>
<tr>
<td>Q14</td>
<td>NK expanded to natural killer Please correct if necessary.</td>
</tr>
<tr>
<td>Q15</td>
<td>Please confirm that given names and surnames have been identified correctly.</td>
</tr>
</tbody>
</table>
Please provide a better quality of figure 4.

We have typesetted figures 1 and 3 in single column. Please check and confirm.

We have changed the reference citation 51 to 15 and 52 to 51. Please check and confirm.

Thank you for your assistance.