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Anti-melanoma vaccinal capacity of CD11c-positive and -negative cell populations present in GM-CSF cultures derived from murine bone marrow precursors

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ABSTRACT

We have initially shown that DC/ApoNec vaccine can induce protection against the poorly immunogenic B16F1 melanoma in mice. The population of DC obtained for vaccination after 7 days culture with murine GM-CSF is heterogeneous and presents about 60% of CD11c+ DC. Therefore, our purpose was to identify the phenotype of the cells obtained after differentiation and its immunogenicity once injected. DC were separated with anti-CD11c microbeads and the two populations identified in terms of CD11c positivity (DC+ and DC−) were also studied. Approximately 26.6% of the cells in DC+ fraction co-expressed CD11c+ and F4/80 markers and 75.4% were double positive for CD11c and CD11b markers. DC+ fraction also expressed Ly6G. DC− fraction was richer in CD11c-/F4/80+ macrophages (44.7%), some of which co-expressed Ly6G (41.8%), and F4/80-/Ly6-G+ neutrophils (34.6%). Both DC+ and DC− fractions displayed similar capacity to phagocyte and endocyte antigens and even expressed levels of MHC Class II and CD80, CD83 and CD86 costimulatory molecules similar to those in the DC fraction. However, only DC/ApoNec vaccine was capable to induce protection in mice ($p < 0.01$). After 24 h co-culture, no detectable level of IL-12 was recorded in DC/ApoNec vaccine, either in supernatant or intracellularly. Therefore, the protection obtained with DC/ApoNec vaccine seemed to be independent of the vaccine's ability to secrete this inflammatory cytokine at the time of injection. In conclusion, we demonstrated that all cell types derived from the culture of mouse bone marrow with GM-CSF are necessary to induce antitumor protection *in vivo*.

1. Introduction

Myeloid dendritic cells (DC) are professional antigen-presenting cells (APCs). Their capacity to capture and present Ags to naïve T lymphocytes (TLs), have positioned them as promising tools in cell-based cancer immunotherapy [1–3].

Different protocols have been designed in order to generate murine DC. These cells are differentiated *in vitro* from bone marrow (BM) progenitors using GM-CSF alone, generating a heterogeneous population of cells, or in combination with IL-4, impairing the development of granulocytes and monocytes [4,5].

DC have the capacity to mature and become immunogenic, increasing their levels of MHC Class I and II, adhesion and co-stimulatory molecules, and secretion of inflammatory cytokines like IL-12, which is essential to mount an immune response mediated by Th1 cells [6]. In immunotherapy murine models, DC were frequently loaded with Ags peptides, whole tumor lysates, apoptotic and necrotic cells or Ags coated with antibodies to target them to DC *via* Fcγ receptors, and it has been possible to induce immune protection and regression of established tumors in different types of cancers [7–16].

Many clinical trials have been designed to evaluate the safety or efficacy of DC-based vaccines. However, the responses obtained to date have been modest [17–27]. Currently, only one autologous cellular vaccine (Sipuleucel-T) has been approved by the FDA for the treatment of prostate cancer; and even so this vaccine only

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generates an increase in patient survival of 4.1 months [28]. Therefore, this promising but modest result underlines that more efforts must be made in order to improve the efficacy of DC-based vaccines.

In recent years, new properties have been attributed to cells that were initially associated only with the innate immune response [29–32]. Increasing evidence suggests that neutrophils, basophils and even eosinophils may participate in the regulation of adaptive immune responses [33–37].

We have initially shown that BM derived DC, loaded with apoptotic and necrotic cells (DC/ApoNec) can induce protection against poorly immunogenic tumors in mice [11]. The population of DC obtained for vaccination is heterogeneous and presents about 60% of CD11c+ DC. Therefore, our purpose was to identify the phenotype of the cells obtained after differentiation with GM-CSF, and its immunogenicity *in vivo* once injected. Here we show results in relation to the phenotypic and functional properties after cell separation in terms of CD11c positivity. Endocytic and phagocytic capacity, cytokine production and immunostimulating potency *in vitro*, and immune protection *in vivo*, were compared to the original heterogeneous population.

2. Materials and methods

2.1. Animals and cell line

8–10-Week-old male C57BL/6 (HH-2^b) and BALB/c mice were obtained from the University of La Plata, Argentina. Mice were maintained in pathogen-free conditions, and studies were performed in accordance with local ethical guidelines. The B16-F1 line was maintained in culture in DMEM (Sigma–Aldrich, MO) supplemented with 10% heat-inactivated FBS (Natocor, Argentina), 100 U/ml penicillin and 100 μg/ml streptomycin (Life Technologies, Gaithersburg, MD). The line was periodically tested to be mycoplasm-free.

2.2. Induction of apoptosis and necrosis

After reaching 70–80% confluence, tumor cells were harvested and resuspended in DMEM for irradiation at 70 Gy (Siemens lineal accelerator). Irradiated cells were stored in liquid nitrogen until use. Irradiated cells were thawed and cultured for 48 h.

Generation of DC, purification and co-culture with apoptotic and necrotic tumor cells (ApoNec)

DC were cultured as previously described [38]. On day 7, DC were harvested. A fraction remained unsorted and the rest was magnetically labeled with anti-CD11c microbeads (Miltenyi Biotec, Germany). The retained fraction (DC+) was separated from the eluted (DC−) by positive selection. DC, DC+ and DC− cell fractions were assayed for *in vitro* analysis and co-cultured with ApoNec cells in a 1/1 ratio for 24 h in the DC conditioned medium.

2.3. Mice vaccination

2×10^5 DC/ApoNec, DC+/ApoNec, DC−/ApoNec or vehicle (PBS) were injected s.c. once a week for 4 weeks in the left flank. The B16 challenge (1.3×10^4 viable cells) was administered on the right flank one week after the last vaccine. Groups of ten mice per vaccinated group were used. Animals were monitored for tumor growth and killed when tumors displayed a size of 400 mm^3.

2.4. Flow cytometric analysis

DC, DC+ and DC− cell fractions were blocked with goat serum or anti-mouse CD16/32 (clone 2.4G2), (BD Bioscience, NJ). Cells were incubated with primary monoclonal antibodies (mAbs): CD11c-PE (HL3), Ly6G-PE (1A8), I-A^b (AF6-120.1), CD80 (1G10/B755), CD83-APC (Michel-19), CD86 (GL1), (all from BD Biosciences); F480 (CI:A3-1), (AbD Serotec, Germany) or rat anti-mouse CD11b hybridoma supernatant. For indirect immunofluorescence, incubation with primary mAbs was followed by the secondary Ab: polyclonal goat anti-mouse immunoglobulins-FITC (DakoCytomation, Denmark) or goat anti-rat cy5 conjugated IgG (Jackson, PA). Cells were fixed in 2% paraformaldehyde and analyzed using a FACS ARIA flow cytometer. Isotype-matched irrelevant mAbs were used as negative controls.

2.5. Phagocytosis assay

After irradiation, ApoNec cells were dyed using PKH26 (Sigma–Aldrich, MO) and co-cultured with DC, DC+ or DC− cell fractions, that were dyed with CFSE (Sigma Aldrich, MO). After 24 h, were fixed and analyzed by flow cytometry. Phagocytosis of ApoNec was defined by the percentage of double-positive cells.

2.6. Endocytosis assay

DC, DC+ and DC− cell fractions, with or without LPS (Sigma–Aldrich, MO) (2 μg/ml) for 24 h, were incubated with FITC-dextran (Invitrogen, CA) (1 μg/μl), at 37 °C or 0 °C for 30 min. The uptake was stopped with PBS. Cell fractions were fixed and analyzed by flow cytometry.

2.7. Mixed lymphocyte reaction (MLR)

DC, DC+ and DC− cell fractions, co-cultured or not co-cultured with ApoNec cells, were treated with mitomycin C (Delta Farma, Argentina) (20 μg/ml), and used as stimulators cells. Effector allogeneic TLs were obtained from lymph nodes of BALB/c mice. Three stimulator/effector ratios were used (1/10, 1/100 and 1/1000), maintaining effectors constant at 2×10^5 cells/well. Concavalin A (3 μg/ml) was used as positive control. After 3 days, ^{3}H-thymidine (0.5 μCi/well) were added to the MLR co-cultures and incubated overnight. The cells were harvested with a Nunc Cell Harvester 8 (Nalge Nunc International Corp., USA) and the ^{3}H-thymidine uptake was determined with a liquid scintillation counter.

2.8. Cytokine production

IL-12 and IL-10 secretion was measured in the supernatants derived from DC/ApoNec, DC/ApoNec plus IFN-γ (1000 U/ml) + LPS (1 μg/ml), DC alone or DC plus IFN-γ (1000 U/ml) + LPS (1 μg/ml), using an enzyme-linked immunosorbent assay (ELISA) with the corresponding BD OptEIA™ Set Mouse (BD Bioscience, NJ). Samples of supernatant were taken every 6 h over a period of 24 h. For detection of intracellular cytokine reservoirs, DC, DC/ApoNec and ApoNec cells were treated with Brefeldin A (Sigma–Aldrich, MO), fixed, permeabilized and incubated with mAbs: rat anti-mouse IL-12-APC (p40/p70) or IL-10-APC (JES5-16E3), (BD Biosciences, NJ). Isotype-matched irrelevant mAbs were used as negative controls.

2.9. Statistical analysis

Data derived from morphological characterization were analyzed by a contingency table (Chi^2 statistic). For statistical analysis of the other experiments One-Way ANOVA of variance and Tukey's Comparison Test were used. Differences were considered significant when $p < 0.05$.

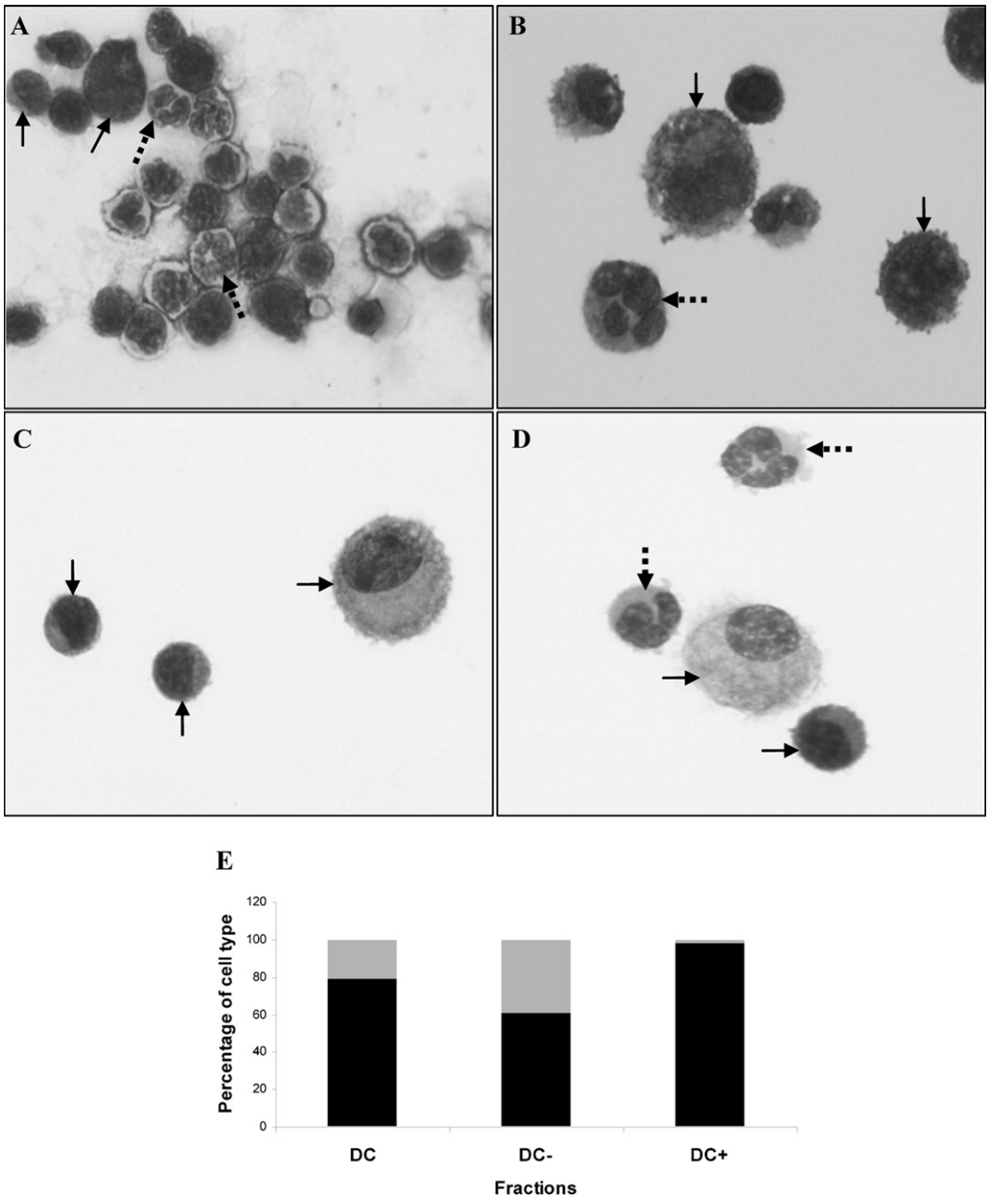

Fig. 1. Cell morphologies by May-Grünwald Giemsa staining. Neutrophils (dotted arrows) and mononuclear cells (full arrows) morphologies present in BM precursors, obtained from femurs and tibias (A), DC (B), DC+ (C) and DC− (D) fractions. Original magnification 1000×. (E) Percentage of neutrophils (gray bars) *vs*. mononuclear cells (black bars) in each fraction. Counts were performed on one hundred cells per smear. $Chi^2 < 0.0001$.

3. Results

3.1. Fractions DC, DC+ and DC− differ in their constitutive proportion of neutrophils and cells of the mononuclear system

DC were differentiated *in vitro* from BM precursors in the presence of GM-CSF. After separation with anti-CD11c microbeads, two populations identified in terms of CD11c positivity were also studied: DC+ and DC−. The morphology of BM precursors and cells included in each fraction were analyzed by May-Grünwald Giemsa staining.

BM cells contained a large variety of precursors, some of which presented a segmented nucleus typical of mouse neutrophils (Fig. 1A). After 7 days culture with GM-CSF and immunomagnetic separation, the DC+ fraction was up to 98% richer in mononuclear cells, while almost all neutrophils contained in the unpurified DC population were collected in the DC− fraction (40%) (Figs. 1B–D). Differences in percentages among DC, DC+ and DC− were statistically significant, $Chi^2 < 0.0001$ (Fig. 1E).

3.2. Determination of surface Ags in DC, DC+ and DC− cell fractions

Cells from DC, DC+ and DC− fractions were incubated with the corresponding Abs and analyzed by flow cytometry.

As expected, the percentage of CD11c+ staining was higher in the DC+ fraction (mean ± SD, 77.6 ± 15.1%) and differed significantly from unpurified DC (mean ± SD, 50.3 ± 8%; $p < 0.01$) and from DC− (mean ± SD, 15.4 ± 4.2%; $p < 0.001$) (Supplementary Fig. S1).

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2012.10.114.

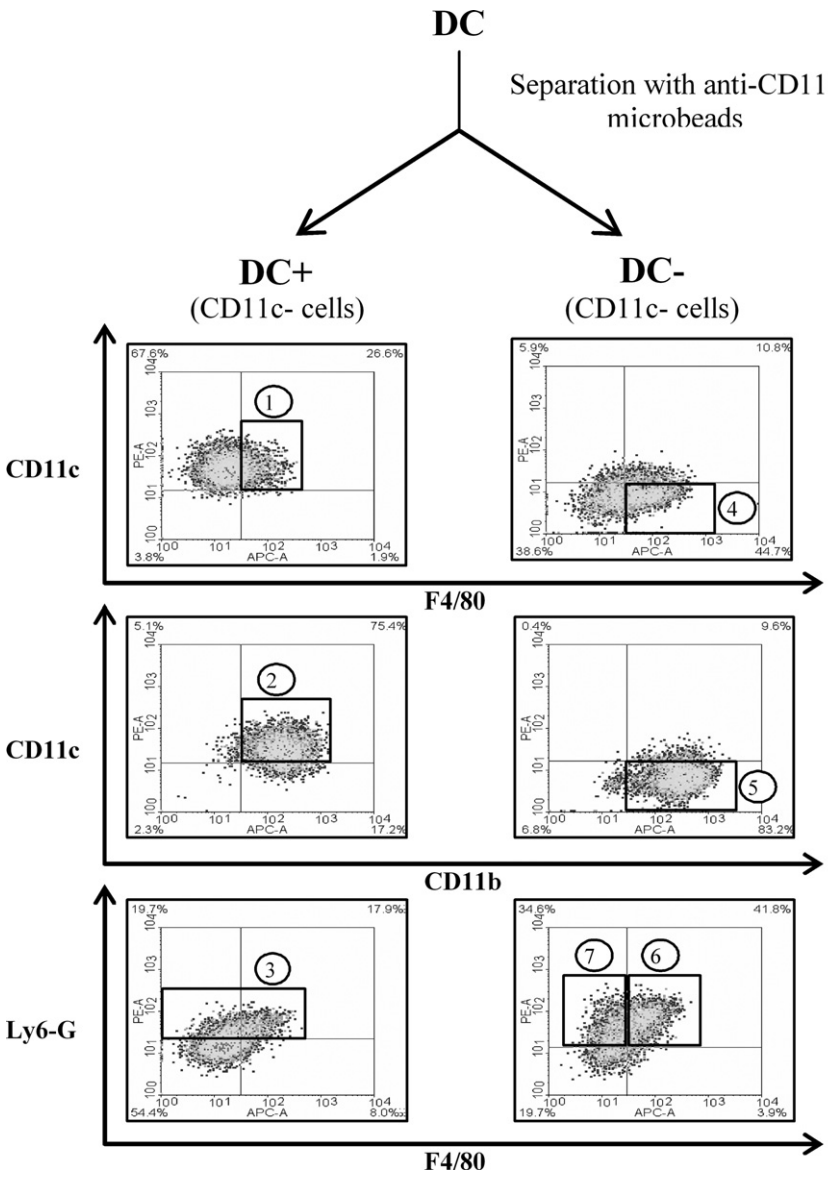

Fig. 2. Surface antigens in DC+ and DC− cell fractions. After separation with CD11c microbeads, cells were incubated with anti-mouse CD11c, F4/80, Ly6G and CD11b mAbs and analyzed by flow cytometry. Density plots for double determinations are shown. 1: CD11c+/F4/80+ DC; 2: CD11c+/CD11b+ DC; 3: CD11c+/Ly6-G+ DC; 4: CD11c-/F4/80+ macrophages; 5: CD11c-/CD11b+ cells; 6: CD11c-/F4/80+/Ly6-G+ macrophages; 7: CD11c-/F4/80-/Ly6-G+ neutrophils. Results are representative of 2–10 experiments.

Approximately 26.6% of the cells included in DC+ fraction co-expressed CD11c+ and F4/80 markers, and 75.4% were double positive for CD11c and CD11b markers (Fig. 2). The DC+ fraction also expressed Ly6G (Fig. 2), a marker typically found in granulocytes. The CD− fraction was richer in CD11c-/F4/80+ cells (44.7%), a phenotype attributable to macrophages, some of which co-expressed Ly6G (41.8%). Cells with a F4/80-/Ly6-G+ phenotype (34.6%) presented characteristics of neutrophils (Fig. 2), according to literature [39].

Similar MCH Class II, CD80, CD83 and CD86 levels were expressed by both DC− and DC+ fractions (Supplementary Fig. S2).

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2012.10.114.

3.3. Uptake efficiency of DC, DC+ and DC− cell fractions

The capacity of cells in each fraction to phagocyte ApoNec cells and to endocyte particles of FITC-dextran was examined.

DC (24.5%), DC+ (27%) and DC− (31.8%) presented similar efficacy to phagocyte ApoNec cells (Supplementary Fig. S3), and to endocyte FITC-dextran particles: DC (59.5%), DC+ (48.1%) and DC− (57.9%) (Supplementary Fig. S3). After incubation with LPS, the endocytic capacity diminished statistically in all fractions compared to those unexposed to LPS, $p < 0.001$ (data not shown), demonstrating that cells are not completely mature after 7 days culture with GM-CSF, and are still capable of reacting to maturative stimuli.

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2012.10.114.

3.4. Allostimulatory activity (MLR)

DC, DC+ and DC− cell fractions co-cultured or not co-cultured with ApoNec, were used as stimulators cells. Effector allogeneic TLs were obtained from lymph nodes of BALB/c mice.

All fractions were capable of stimulating naïve TLs as compared to unstimulated ones (medium *vs.* DC $p < 0.001$; medium

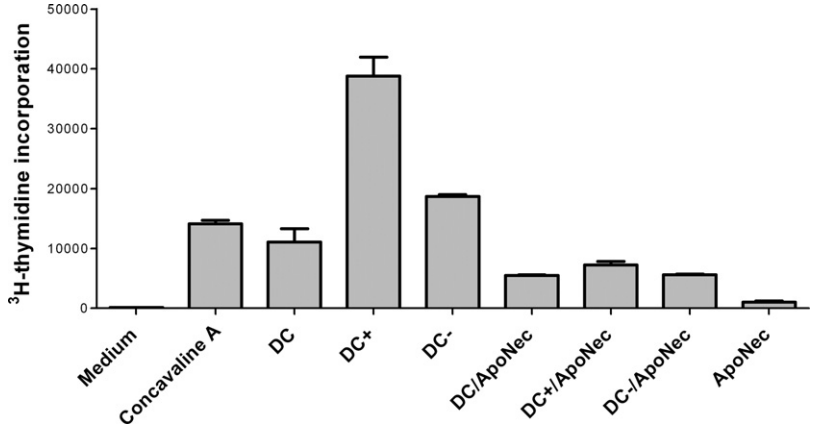

Fig. 3. Mixed lymphocyte reaction. DC, DC+ and DC− cell fractions, co-cultivated or not co-cultivated with ApoNec cells, were treated with Mitomycin C and used as stimulator cells of allogeneic naïve TLs. ^{3}H-thymidine was added 18 h before harvesting. Concavalin A (3 μg/ml) was used as positive control. One of 2 representative experiments, with stimulator/effector ratio of 1/10 is shown.

vs. DC+ $p < 0.001$; medium *vs.* DC− $p < 0.001$); (ApoNec *vs.* DC $p < 0.01$; ApoNec *vs.* DC+ $p < 0.001$; ApoNec *vs.* DC− $p < 0.001$), being DC+ the most powerful stimulator (DC+ *vs.* DC $p < 0.001$; DC+ *vs.* DC− $p < 0.001$). After co-culture, DC+ and DC− diminished their stimulatory capacity (DC+ *vs.* DC+/ApoNec $p < 0.001$; DC− *vs.* DC−/ApoNec $p < 0.01$), while remaining statistically the same in DC (Fig. 3).

3.5. DC+ and DC− fractions cooperate to confer protection in vivo

As DC is a heterogeneous population, previously reported immunity in vaccinated mice might have been exclusively due to CD11c+ subpopulation. In order to determine whether CD11c+ DC were the sole responsible for protection *in vivo*, mice were vaccinated s.c. weekly with DC/ApoNec, DC+/ApoNec or DC−/ApoNec and challenged one week later. Only the vaccine generated with the unselected DC (DC/ApoNec) was capable of protecting the animals significantly from the challenge with melanoma viable cells (Fig. 4).

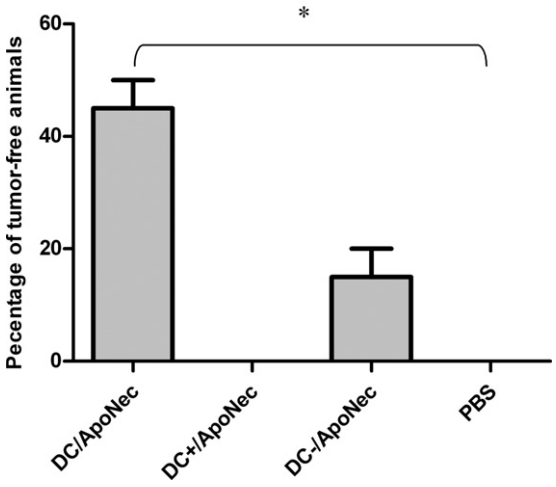

Fig. 4. *In vivo* protection assay. After completion of the vaccination schedule and challenge, the mice were monitored every day for tumor growth over 14 weeks. An average of 2 experiments (twenty animals per each vaccinated group) is shown. The mean percentage of tumor-free animals vaccinated with DC/ApoNec after 14 weeks was 45%, DC+/ApoNec: 0%, DC−/ApoNec: 15% and PBS: 0%. (*) DC/ApoNec *vs.* PBS, $p < 0.01$, DC/ApoNec *vs.* DC+/ApoNec, $p < 0.01$; DC/ApoNec *vs.* DC−/ApoNec, $p < 0.05$.

3.6. DC/ApoNec vaccine does not secrete IL-12 at the time of administration and does not have intracellular reservoirs

In order to determine if DC/ApoNec vaccine efficacy could be related to an active secretion of IL-12 by the phagocytic cells contained in the heterogeneous vaccine at the time of administration to animals, secretion of this cytokine was determined in the supernatants derived from DC/ApoNec, DC/ApoNec plus IFN-γ + LPS, DC alone or DC plus IFN-γ + LPS, at regular intervals over a period of 24 h.

DC alone and DC/ApoNec vaccine did not secrete detectable levels of IL-12 over 24 h. In contrast, when either DC or DC/ApoNec were incubated with IFN-γ + LPS, a high level of IL-12 was recorded, but only in the first 6 h of co-culture (mean ± SD, 1430 ± 73 pg/ml and 621 ± 17.4 pg/ml respectively). Thereafter, the secretion decreased to undetectable levels in 24 h (Fig. 5A).

In addition, IL-10 was not detectable in the supernatants of either DC or DC/ApoNec vaccine over 24 h. However, when DC/ApoNec was incubated with IFN-γ + LPS, a low quantity of IL-10 was secreted, but also only in the first 6 h of co-culture (mean ± SD, 38.9 ± 12.2 pg/ml), and became undetectable after 24 h (data not shown).

Moreover, to explore whether DC/ApoNec vaccine has the ability to synthesize IL-10 and/or IL-12, intracellular detection of cytokines was performed by flow cytometry. As seen in histograms (Fig. 5B), neither DC/ApoNec nor DC have intracellular reservoirs of such cytokines.

4. Discussion

We found that murine BM precursors cultured for 7 days with GM-CSF allowed the development of a heterogeneous population enriched in CD11c+ DC, with a significant proportion of neutrophils and macrophages.

Certain authors have identified morphologically and even functionally mature neutrophils in mouse BM [40], which is partly consistent with our observations after May-Grünwald Giemsa staining. Although it is known that these cells have a short survival period in blood, it was not surprising to find them in our cultures. GM-CSF generates anti-apoptotic signals that prevent neutrophil apoptosis [41], and even stimulate an increase in the expression of activation markers like CD11b [42]. However, this marker is also expressed in other cells belonging to the myeloid lineage [43,44], thus explaining the high percentage of CD11b we have found in all cell fractions.

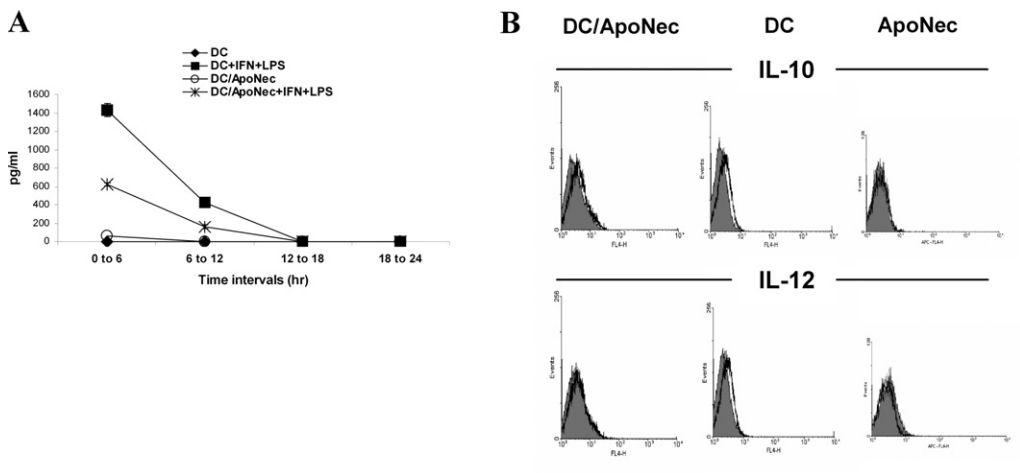

Fig. 5. Secretion of IL-12 over a period of 24 h (A). Intracellular reservoirs of IL-10 and IL-12 after 24 h of culture (B). Empty histograms represent the isotype control. In each case (A) and (B), 1 of 2 representative experiments is shown.

After separation with anti-CD11c microbeads, macrophages and neutrophils were selected in the DC− fraction, representing a 40% of the total number of cells evidenced by May-Grünwald Giemsa staining. It should be noted that this result was coincident with those obtained employing anti-Ly6-G mAbs (34.6%). Likewise, the percentage of neutrophils identified in DC fraction by nuclear staining (20%) and mAbs (26.6%), was also similar. In contrast, in the DC+ fraction only 2% of the total number of cells was identified as neutrophils by their typical nuclear morphology, while 19.7% expressed Ly6-G. This suggests that the remaining percentage of Ly6-G expressed in this fraction is associated with CD11c+ DC.

Cells belonging to the mononuclear system and granulocytes have many common features as a consequence of deriving from committed BM progenitors [45]. Thus, they share some markers. In DC and DC+ we have identified a subpopulation of cells that expressed both CD11c and F4/80. It has been shown that certain myeloid cells co-express these two markers and display a

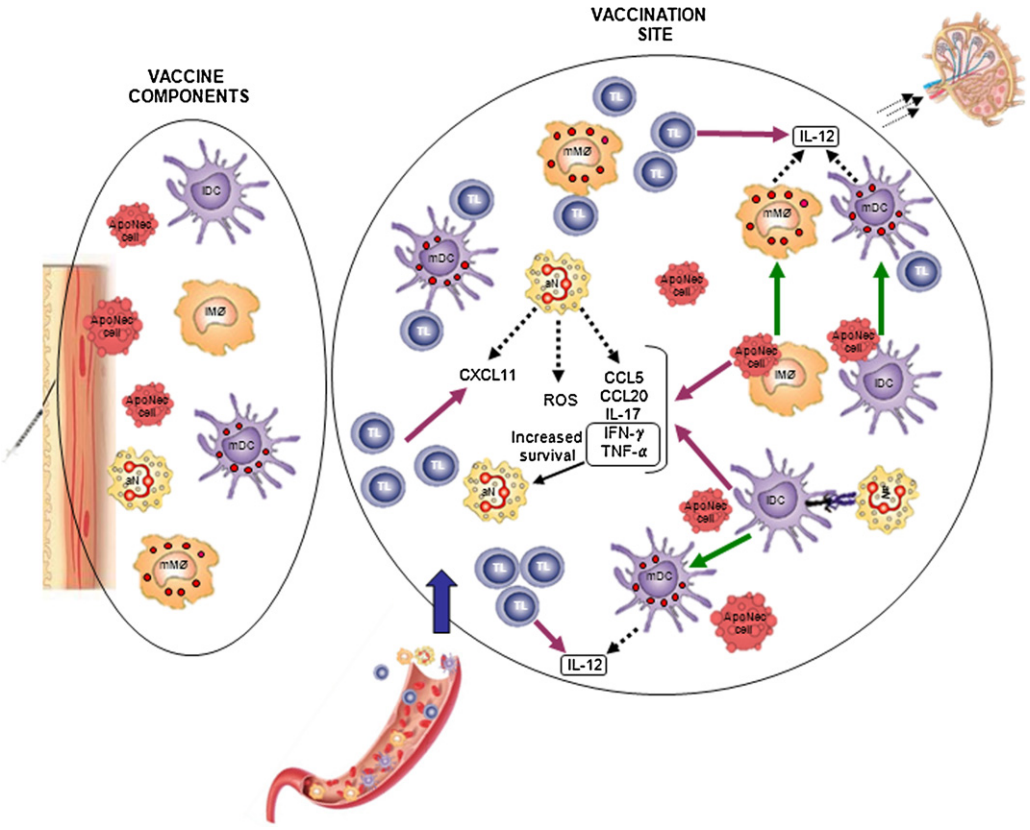

Fig. 6. *In vivo* mechanism of interaction between DC/ApoNec vaccine components and host immune system. Vaccination site: activated neutrophils (aN) release factors that attract and activate macrophages, DC CD11c+ and TLs from the host immune system. These factors increase survival of neutrophils, that induce DC maturation *via* glycosylation-dependent interactions. Immature DC CD11c+ (iDC) and macrophages (iMø) phagocytes ApoNec cells, mature and secrete IL-12. APCs that have been matured *in vitro* and *in vivo* interact with TLs. Some APCs go to secondary lymphoid organs. mDC: mature DC; mMø: mature macrophage; ROS: reactive oxygen species. Dotted arrows: secretion; green arrows: differentiation; pink arrows: recruitment. (For interpretation of the references to color in the artwork, the reader is referred to the web version of the article.)

phenotype that some authors prefer to classify as "dendritic cells that still express F4/80" [46], while others prefer to categorize them as "macrophages expressing CD11c" [47].

Furthermore, we identified a subpopulation that co-expresses F4/80 and Ly6-G markers. Some authors have found double-positive myeloid cells (F4/80+ Ly6G+) in the peritoneal cavity of mice in response to systemic bacterial infections after treatment with monophosphoryl lipid A. This population has characteristics consistent with the monocyte/macrophage lineage [39].

After separation by CD11c expression, DC+ and DC− fractions displayed similar capacity to phagocyte and endocyte Ags, and expressed similar levels of MHC Class II, CD80, CD83 and CD86. Certain evidence tends to highlight CD11c+ DC as professional APCs for primary T-cell responses, and macrophages as APCs that participate in amplifying the immune responses initiated by them. However, increasing evidence suggests that neutrophils may communicate with TLs and even signal DC, inducing its maturation [29–31,33–35].

Considering that all the above mentioned cell subtypes were present in the DC fraction, with similar capacities of uptake and potential ability to cooperate with one another, it is explainable that vaccination with DC/ApoNec generates better protection than DC+/ApoNec or DC−/ApoNec.

DC+ cells were the most powerful stimulator of TLs. But strikingly, after co-culture, DC+ and DC− diminished their stimulatory capacity while this did not happen with unselected DC. It has been reported that apoptotic cells can secrete immunosuppressive cytokines such as IL-10 as they die, and do not induce MLR stimulatory activity [48,49]. In light of this evidence, we wanted to rule out that our mixture of ApoNec cells were not secreting IL-10 after 24 h culture. No detectable levels of this cytokine were found (data not shown), indicating that the decrease in the MLR stimulatory activity after co-culture DC+ and DC− with ApoNec cells was not due to the above mentioned possibility.

In addition, as no detectable level of IL-12 was recorded, either in supernatant or intracellular, it was considered that the protection obtained was independent of the vaccine ability to secrete this cytokine at the time of being injected into animals.

In a previous study we found that both CD4+ and CD8+ TLs were required for the antitumor response observed [11]. Therefore, we suggest that the shift of the balance Th1/Th2 to an inflammatory Th1 response occurred after completion of the vaccination schedule and at the vaccination site.

We previously identified a neoformation of tertiary lymphoid tissue at the site of DC/ApoNec administration [38]. Then, it would be possible that DC+/ApoNec and DC−/ApoNec vaccines lack the ability to generate that structure, altering their ability to induce an effective immune response *in vivo*.

In conclusion, we have demonstrated that all cell types derived from the culture of mouse BM with GM-CSF are necessary to induce antitumor protection *in vivo*.

We have hypothesized (Fig. 6) that in the vaccination site, activated neutrophils derived from the vaccine would be releasing factors [30,34,35,50–52], which would be attracting and activating macrophages, DC and TLs from the host immune system.

In the presence of ApoNec cells, neutrophils would be inducing DC maturation *via* glycosylation-dependent interactions between Mac-1 and DC-SIGN [33,53–55].

Immature DC CD11c+ and macrophages could be phagocyting ApoNec cells *in vivo*, secreting IL-12 and attracting more TLs.

APCs that have been matured *in vitro* and *in vivo* may be interacting with TLs reaching the vaccination site. However, some APCs would travel to secondary lymphoid organs to initiate the activation of naive TLs.

In conclusion, we present a model by which every cell present in the vaccine are important to induce *in vivo* protection to mice.

The implications of this model in human vaccination with DC may be important, since in humans, DC are generally derived from blood circulating monocytes and matured *in vitro* by the addition of GM-CSF and IL-4. It would be important to determine if the presence of other myeloid cells such as those described in this paper would increase the vaccine efficiency.

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References

[1] Lesterhuis WJ, de Vries IJM, Adema GJ, Punt CJA. Dendritic cell-based vaccines in cancer immunotherapy: an update on clinical and immunological results. Ann Oncol 2004;15(4):iv145–51.
[2] Delamarre L, Mellman I. Harnessing dendritic cells for immunotherapy. Semin Immunol 2011;23:2–11.
[3] Timmerman J, Levy R. Dendritic cell vaccine for cancer immunotherapy. Annu Rev Med 1999;50:507–29.
[4] O' Neill HC, Wilson HL. Limitations with in vitro production of dendritic cells using cytokines. J Leukoc Biol 2004;75:600–3.
[5] Morelli AE, Zahorchak AF, Larregina AT, Colvin BL, Logar AJ, Takayama T, et al. Cytokine production by mouse myeloid dendritic cells in relation to differentiation and terminal maturation induced by lipopolysaccharide or CD40 ligation. Blood 2001;98(5):1512–23.
[6] Reis e Sousa C. Dendritic cells in a mature age. Nature 2006;6:476–82.
[7] Mayordomo JI, Zorina T, Storkus WJ, Zitvogel L, Celluzi C, Falo LD, et al. Bone marrow derived dendritic cells pulsed with syngeneic tumour peptides elicit protective and therapeutic antitumour immunity. Nature Med 1996;1:1297–302.
[8] Hegmans JP, Hemmes A, Aerts JG, Hoogsteden HC, Lambrecht BN. Immunotherapy of Murine Malignant Mesothelioma Using Tumor Lysate-pulsed Dendritic Cells. Am J Respir Crit Care Med 2005;171:1168–77.
[9] Rossowska J, Pajtasz-Piasecka E, Szyda A, Ziêtara N, Duoe D. Tissue localization of tumor antigen-loaded mouse dendritic cells applied as an anti-tumor vaccine and their influence on immune response. Folia Histochem Cytobiol 2007;45(4):349–55.
[10] Chen Z, Moyana T, Saxena A, Warrington R, Jia Z, Xiang J. Efficient antitumor immunity derived from maturation of dendritic cells that had phagocytosed apoptotic/necrotic tumor cells. Int J Cancer 2001;93:539–48.
[11] Goldszmid RS, Idoyaga J, Bravo AI, Steinman R, Mordoh J, Wainstok R. Dendritic cells charged with apoptotic tumor cells induce long-lived protective CD4+ and CD8+ T cell immunity against B16 melanoma. J Immunol 2003;171:5940–7.
[12] Celluzzi CM, Mayordomo JI, Storkus WJ, Lotze MT, Falo LD. Peptide-pulsed dendritic cells induce antigen-specific, CTL-mediated protective tumor immunity. J Exp Med 1996;183:283–7.
[13] Benencia F, Sprague L, McGinty J, Pate M, Muccioli M. Dendritic cells, the tumor microenvironment and the challenges for an effective antitumor vaccination. J Biomed Biotechnol 2011;2012:1–15.
[14] Zitvogel L, Mayordomo JI, Tjandrawan T, DeLeo AB, Clarke MR, Lotze MT, et al. Therapy of murine tumors with tumor peptide-pulsed dendritic cells: dependence on T cells, B7 costimulation, and T helper cell 1-associated cytokines. J Exp Med 1996;83:87–97.
[15] Shimizu K, Fields RC, Giedlin M, Mule J. Systemic administration of interleukin 2 enhances the therapeutic efficacy of dendritic cell-based tumor vaccines. Proc Natl Acad Sci USA 1999;96:2268–73.
[16] Pilon-Tomas S, Mackay A, Vohra N, Mulé JJ. Blockade of programmed death ligand 1 enhances the therapeutic efficacy of combination immunotherapy against melanoma. J Immunol 2010;184:3442–9.
[17] Vulink A, Radford KJ, Melief C, Hart DN. Dendritic cells in cancer immunotherapy. Adv Cancer Res 2008;99:363–407.
[18] Banerjee DK, Dhodapkar MV, Matayeva E, Steinman RM, Dhodapkar KM. Expansion of FOXP3 high regulatory T cells by human dendritic cells (DCs) in vitro and after injection of cytokine-matured DCs in myeloma patients. Blood 2006;108(8):2655–61.
[19] Von Euw EM, Barrio MM, Furman D, Levy EM, Bianchini M, Peguillet I, et al. Phase I clinical study of vaccination of melanoma patients with dendritic cells loaded with allogeneic apoptotic/necrotic melanoma cells. Analysis of toxicity and immune response to the vaccine and of IL-10-1082 promoter genotype as predictor of disease progression. J Transl Med 2008;6(6).

[20] Aarntzen JG, Figdor CG, Adema GJ, Punt CJ, De Vries JM. Dendritic cell vaccination and immune monitoring. Cancer Immunol Immunother 2008;57(10):1559–68.
[21] Palucka AK, Ueno H, Fay J, Banchereau J. Dendritic cells: a critical player in cancer therapy? J Immunother 2008;31:793–805.
[22] Kalantari T, Kamali-Sarvestani E, Ciric B, Karimi MH, Kalantari M, Faridar A, et al. Generation of immunogenic and tolerogenic clinical-grade dendritic cells. Immunol Res 2011;51(2-3):153–60.
[23] Aptsiauri N, Cabrera T, Garcia-Lora A, Garrido F. Cancer immune escape: implications for immunotherapy. Cancer Immunol Immunother 2012;121:123–19.
[24] Madorsky-Rowdo FP, Lacreu ML, Mordoh J. Melanoma vaccines and modulation of the immune system in the clinical setting: building from new realities. Frontiers in immunology 2012;3:103.
[25] Lesterhuis WJ, de Vries IJ, Adema GJ, Punt CJ. Dendritic cell-based vaccines in cancer immunotherapy: an update on clinical and immunological results. Ann Oncol 2004;15(4):iv145–51.
[26] Nakai N, Hartmann G, Kishimoto S, Katoh N. Dendritic cell vaccination in human melanoma: relationships between clinical effects and vaccine parameters. Pigment Cell Melanoma Res 2010;23:607–19.
[27] Iwashita Y, Tahara K, Goto S, Sasaki A, Kai S, Seike M, et al. A phase I study of autologous dendritic cell-based immunotherapy for patients with unresectable primary liver cancer. Cancer Immunol Immunother 2003;52:155–61.
[28] Sims RB. Development of sipuleucel-T: autologous cellular immunotherapy for the treatment of metastatic castrate resistant prostate cancer. Vaccine 2012;30(29):4394–7.
[29] Culshaw S, Millington OR, Brewer JM, McInnes IB. Murine neutrophils present class II restricted antigen. Immunol Lett 2008;118:49–54.
[30] Abi Abdallah DS, Egan CE, Butcher BA, Denkers EY. Mouse neutrophils are professional antigen-presenting cells programmed to instruct Th1 and Th17 T-cell differentiation. Int Immunol 2011;23(5):317–26.
[31] Beauvillain C, Cunin P, Doni A, Scotet M, Jaillon S, Loiry ML, et al. CCR7 is involved in the migration of neutrophils to lymph nodes. Gen Virol 2010;91:2158–66.
[32] Mikhak Z, Luster AD. The emergence of basophils as antigen-presenting cells in Th2 inflammatory responses. J Mol Cell Biol 2009;1(2):69–71.
[33] Van Gisbergen KP, Sanchez-Hernandez M, Geijtenbeek TB, Van Kooyk Y. Neutrophils mediate immune modulation of dendritic cells through glycosylation dependent interactions between Mac-1 and DC-SIGN. J Exp Med 2005;201(8):1281–92.
[34] Kumar V, Sharma A. Neutrophils: cinderella of innate immune system. Int Immunopharmacol 2010;10:1325–34.
[35] Terrin E, Beaman BL. Interferon-C activation of polymorphonuclear neutrophil function. Immunology 2004;112:2–12.
[36] Perrigoue JG, Saenz SA, Siracusa MC, Allenspach EJ, Taylor BC, Giacomin PR, et al. Major histocompatibility complex class II-dependent basophil-CD4$^+$ T cell interactions promote T_H2 cytokine-dependent immunity. Nat Immunol 2009;10(7):697–705.
[37] Lotfi R, Lotze T. Eosinophils induce DC maturation, regulating immunity. J Leukoc Biol 2008;83:456–60.
[38] Mac Keon S, Gazzaniga S, Mallerman J, Bravo AI, Mordoh J, Wainstok R. Vaccination with dendritic cells charged with apoptotic/necrotic B16 melanoma induces the formation of subcutaneous lymphoid tissue. Vaccine 2010;28:8162–8.
[39] Romero CD, Varma TK, Hobbs JB, Reyes A, Driver B, Sherwood ER. The toll-like receptor 4 agonist monophosphoryl lipid A augments innate host resistance to systemic bacterial infection. Infect Immun 2011;79(9):3576–87.
[40] Boxio R, Bossenmeyer C, Steinckwich N, Dournon C, Nube O. Mouse bone marrow contains large numbers of functionally competent neutrophils. J Leukoc Biol 2004;75:604–11.
[41] Kozo Y, Yukio S, Motoki I, Haruo N, Takashi Y, Atsushi K, et al. Granulocyte macrophage-colony stimulating factor delays neutrophil apoptosis and primes its function through Ia-type phosphoinositide 3-kinase. J Leukoc Biol 2002;72:1020–6.
[42] Costantini C, Micheletti A, Calzetti F, Perbellini O, Pizzolo G, Cassatella MA. Neutrophil activation and survival are modulated by interaction with NK cells. Int Immunol 2010;22(10):827–38.
[43] Gilliet M, Boonstra A, Paturel C, Antonenko S, Xu X, Trinchieri G, et al. The development of murine plasmacytoid dendritic cell precursors is differentially regulated by FLT3-ligand and granulocyte/macrophage colony-stimulating factor. J Exp Med 2002;195:953–8.
[44] Maruyama K, Ii M, Cursiefen C, Jackson DG, Keino H, Tomita M, et al. Inflammation-induced lymphangiogenesis in the cornea arises from CD11b-positive macrophages. J Clin Invest 2005;115(9):2363–72.
[45] Wu L, Liu YJ. Development of dendritic-cell lineages. Immunity 2007;26:741–50.
[46] Miloud T, Hammerling GJ, Garbio N. Review of murine dendritic cells: types, location, and development. Methods Mol Biol 2010;595:21–42.
[47] Hume DA. Macrophages as APC and the dendritic cell myth. J Immunol 2008;181:5829–35.
[48] Steinman RM, Turley S, Mellman I, Inaba K. The induction of tolerance by dendritic cells that have captured apoptotic cells. J Exp Med 2000;191(3):411–6.
[49] Sauter B, Albert ML, Francisco L, Larsson M, Somersan S, Bhardwaj N. Consequences of cell death: exposure to necrotic tumor cells, but not primary tissue cells or apoptotic cells, induces the maturation of immunostimulatory dendritic cells. J Exp Med 2000;191(3):7423–33.
[50] Bennouna S, Bliss SK, Curiel TJ, Denkers EY. Microbial infection activation of dendritic cells during neutrophils instruct recruitment and cross-talk in the innate immune system. J Immunol 2003;171:6052–8.
[51] Megiovanni AM, Sanchez F, Robledo-Sarmiento M, Morel C, Gluckman JC, Boudaly S. Polymorphonuclear neutrophils deliver activation signals and antigenic molecules to dendritic cells: a new link between leukocytes upstream of T lymphocytes. J Leukoc Biol 2006;79:977–88.
[52] Scapini P, Lapinet-Vera JA, Gasperini S, Calzetti F, Bazzoni F, Cassatella MA. The neutrophil as a cellular source of chemokines. Immunol Rev 2000;177:195–203.
[53] Van Gisbergen KP, Geijtenbeek TB, Van Kooyk Y. Close encounters of neutrophils and DCs. Trends Immunol 2005;26(12):626–31.
[54] Park SJ, Burdick MD, Mehrad B. Neutrophils mediate maturation and efflux of lung dendritic cells in response to *Aspergillus fumigatus* germ tubes. Infect Immunol 2012;80(5):1759–65.
[55] Ludwig IS, Geijtenbeek TB, Van Kooyk Y. Two-way communication between neutrophils and dendritic cells. Curr Opin Pharmacol 2006;6:408–13.