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Short Communication

B-1 cells promote immunosurveillance against murine melanoma in host absence of CCR5: New perspective in autologous vaccination therapy

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ABSTRACT

Autologous vaccination with tumor-primed dendritic cells increases immune response against tumor, which seems to be improved in host absence of CCR5. Because B-1 lymphocytes modulate the activity of different immune cells, we decided to study their influence in the resistance against murine B16F10 melanoma in a CCR5 deprived environment. Adoptive transfer of peritoneal B-1 $CCR5^{+/+}$ lymphocytes to $CCR5^{-/-}$ animals inhibited the establishment of lung metastasis and melanoma cell growth, in comparison to saline-treated $CCR5^{-/-}$ mice. *In loco* cell analysis demonstrated that the adoptive transfer of B-1 $CCR5^{+/+}$ lymphocytes to CCR5 deficient host was associated with a more intense influx of T $CD8^{+}$ to tumor site, indicating that the presence of $CCR5^{+/+}$ B-1 cells in the tumor environment induces the migration of T CD8 $CCR5^{-/-}$ cells to the implantation site. To corroborate this idea, $CCR5^{-/-}$ mice were injected with non B-1 peritoneal cells from wild type (WT) mice before B16F10 inoculation. In this regimen, $CCR5^{-/-}$ mice were not protected from tumor growth reinforcing the idea that, in host absence of CCR5, B-1 cells are essential to confer tumor resistance. This work indicates that, in the host absence of CCR5, naive B-1 cells may activate CD8T lymphocytes thereby promoting tumor resistance. Our results strongly suggest that autologous vaccination with B-1 lymphocytes in combination with CCR5 antagonists can be an alternative approach to tumor therapy.

Introduction

Different factors present in the melanoma microenvironment may influence its development and progression. Cytokines, chemokines, growing factors, matrix metalloproteinases and infiltrating inflammatory cells have been associated with tumor progression (Labrousse et al., 2004). Among these factors, chemokines are receiving significant attention due to the modulation of tumor growth dynamics by their ability to induce the migration of inflammatory cells to the tumor site. Although paradoxical, under determined conditions, the presence of inflammatory cells in the tumor sites may favor tumor progression. For example, tumor-associated macrophages (TAMs) can contribute to cancer progression by producing soluble factors that either can promote tumor growth or help tumor cells to evade from host immune-surveillance (Hussein, 2006). In this sense, CCL5, so-called RANTES, is one of the chemokines presents in melanoma milieu and that can contribute to tumor evolution (Mrowietz et al., 1999).

The mechanisms involved in the association between CCL5 and tumor progression remain an object of study, but there is a large body of evidence demonstrating that both endogenous and host secretion of CCL5 are benefit for tumor cells. For example, tumor implantation and local secretion of CCL5 can induce an inflammatory context that favors a local pro-tumoral environment. This

Abbreviations: CCR5, C-C chemokine receptor type 5; CCL5, Chemokine (C-C motif) ligand 5; DC, dendritic cells; EDTA, ethylenediamine tetraacetic acid; ERK, extracellular-signal-regulated kinase; MAPK, mitogen-activated protein; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; RANTES, regulated on activation, normal T cell expressed and secreted; TAM, tumor-associated macrophage; WT, wild type.

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phenomenon seems to be associated with the secretion of regulatory cytokines as TGF-β and IL-10 by TAMs or the migration or differentiation of T regulatory cells, culminating in the generation *in loco* of an immunosuppressive milieu (Solinas et al., 2009; Dannenmann et al., 2013). In addition, the secretion of CCL5 by host cells appears to promote tumor growth *via* the activation of the nuclear transcription factor NF-κB (Song et al., 2012; Lee et al., 2012). In contrast, Ng-Cashin and cols demonstrated that, in host absence of CCR5 the vaccination with tumor-pulsed dendritic cells (DC) protected mice from B16F10 melanoma cells. However, this phenomenon was dependent on CCR5 expression on DC, showing that these cells were attracted to the tumor site via this receptor (Ng-Cashin et al., 2003).

In the context of CCL5 and its association with B16F10 melanoma, our group showed that B-1 cells, a population of non-conventional B lymphocytes, promote melanoma progression by activation of the ERK/MAPK pathway, and CCL5 secretion, in a cell-to-cell-contact dependent manner (Perez et al., 2008; Xander et al., 2013). Thus, we decided to determine the effect of B-1cells in tumor progression in a CCR5 deprived environment.

Our data demonstrate that likewise DC, the CCR5 receptor is involved in B-1 cells chemotaxis in response to melanoma stimulation. In addition, adoptive transfer of naïve B-1 cells to CCR5 deficient mice resulted in the inhibition of B16F10 melanoma growth and lung colonization. Although the mechanisms involved in this phenomenon remain object of investigation, this work highlighted a new role for B-1 lymphocytes and indicated that adoptive transfer of naïve B-1 cells in addition to host CCR5 depletion is a possible alternative to melanoma management.

Materials and methods

Animals

Isogenic male C57Bl/6 wild type (WT) or constitutively expressing green fluorescent protein (GFP) mice 8-12 weeks of age, were obtained from Animal Care Facility at the Federal University of São Paulo (CEDEME). Male C57BL/6 CCR5 knockout mice ($CCR5^{-/-}$) were kindly provided by Prof. João Santana da Silva (USP, Brazil). Mice were housed in a specific pathogen-free facility according to NIH guide for care and use of laboratory animals. All of the procedures were previously reviewed and approved by the internal ethics committee of the institution (CEP1174/07).

B16F10 cell line

All experiments were performed with B16F10 melanoma cells. B16F10 cells were maintained in RPMI (Cultilab, Brazil) supplemented with 2 mM L-glutamine, 10 mM HEPES, 24 mM sodium bicarbonate, 100 units/ml of penicillin, 100 μg/ml of streptomycin and 10% of heat-inactivated fetal calf serum (Cultilab, Brazil). Cells were cultivated under 5% CO_2 atmosphere at 37 °C.

Adoptive transfer of B-1 cells

The peritoneal cavity was washed with PBS plus 0.5% BSA and 2 mM ethylenediamine tetraacetic acid (EDTA) and the cells were incubated with a solution containing anti-CD23 and anti-CD19 antibodies. The B-1 cells were obtained by electrostatic sorting as $CD23^-$ and $CD19^+$ cells (FACS Aria II; BD Biosciences). The B-1 cell population presented a purity of 98.5–99.8%. Thereafter, depending on the experimental protocol, 3.4×10^5 or 1×10^6 B-1 cells were injected, intraperitoneally, 24 h before mice inoculation with B16F10 cells.

Migration assay

To analyze the migration potential of B-1 cell in response to melanoma stimulation, purified B-1 cells from WT or $CCR5^{-/-}$ animals were incubated on the upper chamber of a 8 micron transwell® system (Corning Inc.) containing 200 μl of RPMI. The inside lower chamber was filled with 600 μl of fresh RPMI, B16F10 supernatant or 2×10^3 B16F10 cells in 600 μl of fresh RPMI culture media. The plates were incubated for 24 h, under 5% CO_2 atmosphere at 37 °C. Afterward, both the upper chamber and the lower chamber supernatant were removed. The adherent cells that migrated trough the membrane were collected and submitted to flow cytometer counting (FACS Canto II, BD Biosciences).

Tumor implantation models

To determine the influence of B-1 cells in B16F10 lung colonization, C57Bl/6 WT or $CCR5^{-/-}$ mice were inoculated intravenously with 1×10^5 B16F10 cells. After 15 days, animals were euthanized and pulmonary B16F10 nodules counted. In order to evaluate the effect of B-1 cells on tumor growth mice were injected subcutaneously, in the left flank, with 2.5×10^5 B16F10 melanoma cells. Using a caliper, we measured the major and minor dimensions of the tumor and its volume calculated with the mathematic equation described by Feldman and cols (Feldman et al., 2009).

Analysis of intratumoral cell content

Subcutaneous tumors were excised, weighted and mechanically disrupted using a 70 μm cell strainer with PBS 2 mM EDTA 0.5% BSA. Cell suspensions were incubated with a solution with anti-CD45, anti-CD3, anti-CD4, anti-CD8, anti-CD19, anti-23, anti-NK1.1 and anti-F4/80 antibodies conjugated with different fluorochromes. The analysis of different cell populations by flow cytometry was carried out after exclusion of all $CD45^-$ cells (non-leukocyte cells) (FACS Canto II, BD Biosciences).

Statistical analysis

All of the data were presented as the mean ± the standard deviation (SD) and were representative of at least two independent experiments ($n = 5$–6 animals/group). The differences between the groups were evaluated using analysis of variance (ANOVA) and Tukey's test as a post-hoc test. Survival rate were evaluated by Mantel-Cox and Gehan-Breslow-Wilcoxon tests. We considered the differences to be significant when the P value was less than 0.05. All of the statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software, USA).

Results

B-1 cells migrate in response to B16F10 stimuli in a CCR5-dependent manner

Although the production of chemokines, as CCL5, by B16F10 cells supports the idea of inflammatory cells chemoattraction to melanoma milieu, there is no evidence of CCR5 expression in B-1cells. The B-1 peritoneal lymphocytes were characterized as $CD19^+/CD23^-$ cells (Fig. 1A, upper panel). The flow cytometric analysis showed that, in the steady state, 10–14% of total B-1 cells express CCR5 on surface (Fig. 1A, lower panel), suggesting that CCL5 is implicated in B-1 cell homing to the tumor site. In order to test this