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Identification of myeloid-derived suppressor cells and T regulatory cells in lung microenvironment after Urethane-induced lung tumor

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ABSTRACT

Tumor growth occurs by the imbalance between cells with effector function and cells with suppressor/regulatory functions. To investigate this scenario we administered the chemical carcinogen Urethane in BALB/c mice and followed these animals during 120 days to observe lung tumor development. In another set of experiments the same protocol was performed with the harvest of spleen, lung and blood at 20 and 30 days after Urethane injection. The lung was used for histology, spleen cells were evaluated for IFN-γ production, and serum nitrite was measured as an indirect form of nitric oxide (NO) evaluation. The spleen and lung-infiltrating cells were evaluated by flow cytometry for $CD11b^{+}Gr\text{-}1^{+}$ myeloid suppressor cells and $CD4^{+}FoxP3^{+}$ T regulatory cells. Urethane led to lung nodules development after 120 days and the time point evaluation showed that splenocytes stimulated *ex vivo* expressed higher levels of IFN-γ 20 days after the chemical injection. Also, the level of nitric oxide in serum was higher after 20 days of Urethane injection. There was no statistical difference in spleen cells percentages for $CD11b^{+}Gr\text{-}1^{+}$ and $CD4^{+}Foxp3^{+}$ in all groups. However, lung-infiltrating cells presented early (20 days) a higher expression of $CD11b^{+}Gr\text{-}1^{+}$ suggesting suppression at this site. In conclusion, it was possible to observe two distinct events at the very early time point after Urethane injection. In periphery there was an increase at the effector immune response (as depicted by IFN-γ-producing cells) and in tumor development site there was an increase at the suppressor cell ($CD11b^{+}Gr\text{-}1^{+}$) phenotype. Suppressor/regulatory cells are targets for cancer therapy.

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1. Introduction

Mouse lung tumors induced by chemical carcinogens present similarities in the gene expression profiles when compared with human adenocarcinoma supporting the utility of tumor mice models [1].

BALB/c mice are classified as intermediate susceptible to Urethane-induced lung tumor as they develop tumors with low multiplicity (2 tumors/mouse). This intermediate susceptibility has been associated with the presence in BALB/c mice of both sensitive Kras polymorphism and a unique Pulmonary adenoma resistance (Par2) allele that down-modulates their neoplasic predilection [2]. Our group showed recently that Urethane injection in BALB/c mice leads to lung tumor development after four months. Lung nodules (2 to 4 in each mouse) presented size range from 0.4 mm^2 to 9 mm^2 and cells with a high ratio of proliferation/apoptosis. However, these mice showed no changes at percentages of $CD4^{+}$ cells in spleen or lymphocytes in blood at this period [3,4]. These findings suggest that the lymphocytes function instead of their number could have changed during the lung tumor development and also that the major changes in the immune system might occur at the early periods after Urethane injection.

The activation of the immune system during cancer development leads cells to secrete factors which improve the anti-cancer response. Redente et al. [5] showed that IFN-γ is essential to reduce lung tumor growth induced by Urethane injection. On the other hand, nitric oxide (NO) produced during inflammation has been associated with early events in the tumorigenic process [6,7]. As NO is highly reactive and unstable in vivo, nitrite/nitrate in serum is most often used as a measure of NO production in blood stream [8]. Nitrite/nitrate higher levels have been associated with poor survival rate in patients with lung cancer [9].

Suppressor cells ($CD11b^{+}Gr\text{-}1^{+}$) and T regulatory cells ($Foxp3^{+}$) have been shown to facilitate tumor development.

The injection of MCA205 (fibrosarcoma) cells in C57BL/6 mice caused an increase in $CD11b^{+}Gr\text{-}1^{+}$ expression in spleen cells [10]. In addition, the transfer of the spleen cells from tumor-bearing mice severely inhibited the generation of tumor-immune T cells in draining lymph node. Gabrilovich et al. [11] found that BALB/c mice inoculated with MethA sarcoma cells developed tumor (1.5 cm in diameter) in 4–5 weeks. The evaluation of splenocytes in this period showed that

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control BALB/c presented 2.7 ± 0.4 Gr-1^+ cells whereas MethA sarcoma-bearing BALB/c mice had a 5 fold increase in Gr-1^+ expression ($14.5 \pm 1.3\%$). CD4-mediated T cell response was not impaired *in vitro* when cocultured with Gr-1^+ whereas CD8-mediated immune responses decreased *in vitro* and *in vivo*.

Granville et al. [12] showed that A/J mice exposed to a tobacco carcinogen increased the lung-associated Foxp3^+ cells. K-Ras mutations are necessary for tobacco carcinogen-driven lung tumorigenesis and mutant K-Ras transgenic mice lacking Foxp3^+ cells developed 75% fewer lung tumors than wild type littermates. Therefore, authors concluded that Foxp3^+ regulatory T cells are required for K-Ras-mediated lung tumorigenesis in mice.

In order to further investigate the mechanisms associated with Urethane-induced lung tumor in BALB/c mice it was our aim in the present study to compare the findings in the lung (nodule numbers and size) with possible alterations in periphery such as IFN-γ-producing cells, NO in serum and CD4^+Foxp3^+ and CD$11b^+$Gr-1^+ cell populations. Our results could lead to anti-tumor therapies based in the modulation of the suppressive factors which may become a potentially effective approach in cancer.

2. Materials and methods

2.1. Animals and experimental design

Eight to 10-week-old male BALB/c mice (bred in CEDEME-UNIFESP) were placed in cages and cared for in accordance with the Principles of Laboratory Animal Care (NIH publication 86-23, revised 1985) and the regulations of the Brazilian Committee on Animal Experimentation. Urethane (Sigma Chemical Company, St Louis, MO) dissolved in 0.9% NaCl was injected in two doses i.p. (1.5 g/kg each dose) with the interval of 48 h. Mice were evaluated 20 days, 30 days and 120 days after Urethane injection. In these evaluation periods mice were anesthetized with Xylazine (Agribrands, Brazil) and Ketamine (Vetbrands, Brazil) diluted in 10 ml of sterile PBS (phosphate buffered solution-OXOID LTD Hampshire England), the abdomen and thorax were opened for the harvesting of spleen, blood and lung.

2.2. Flow cytometry

Spleen and lung single cell suspensions were prepared by pressing each organ through a 400 μm sterile nylon mesh. The spleen and lung single cell suspensions were placed in individual tubes and submitted to 1 min of distilled water to cause hemolysis. For lung evaluations using flow cytometry we used 6 naïve BALB/c mice and pooled 3 of them for each experiment since we obtained few cells from the lung of non-manipulated naïve mice. Using this approach we obtained data from 2 individual and different experiments.

For surface markers, 1×10^6 cells from the spleen or lung were incubated for 20 min with the rat anti-mouse (BD Biosciences Pharmingen) CD4 PerCP, CD3 APC, CD11b APC, Gr-1 Pe (Immuno-Tools, Germany). Cells were washed and fixed with 4% (v/v) paraformaldehyde in PBS for 20 min at room temperature.

For Foxp3 intracellular staining, 1×10^6 cells from the spleen or lung were permeabilized with PBS containing 0.5% BSA (v/v) and 0.1% (v/v) saponin and incubated with the rat anti-mouse Foxp3 FITC (eBioscience — San Diego, CA) for 30 min at room temperature. Cells were washed twice with PBS containing 0.5% BSA (v/v) and 0.2% (v/v) saponin, fixed with 4% (w/v) paraformaldehyde and analyzed by flow cytometry.

IFN-γ-producing cells were identified *ex vivo* using cell suspension from spleen (1×10^6) suspended in RPMI 1640 medium (Sigma-Aldrich Brasil Ltda) and cultured in a test plate (TPP/Switzerland) in the presence or absence of the activators (Sigma-Aldrich Brasil Ltda) PMA (50 ng/ml), ionomycin (1 μg/ml) and a protein transport inhibitor brefeldin A (1 μg/ml) in humidified incubator containing 5% of CO_2 at 37 °C for 4 h. Cells were washed twice in ice-cold PBS (Sigma-Aldrich Brasil Ltda), overnight fixed, and permeabilized with 0.2% Triton X-100 (6 min at 37 °C). Then, cells were washed twice in ice-cold PBS, pelleted and stained (30 min at 4 °C) for intracellular cytokine using anti-mouse IFN-γ Alexa-Fluor (eBioscience — San Diego, CA). Cells were washed in PBS and suspended in PBS-BSA for flow cytometry analysis. Cells were analyzed in a FACSCalibur (Cell Quest Pro Software) Cell Cytometer (BD Biosciences). At least 10,000 events were evaluated.

2.3. Nitrite serum measurement

As NO is highly reactive and unstable in vivo, nitrite/nitrate in serum is most often used as a measure of NO production in blood stream (Green et al., 1982). Peripheral blood samples from BALB/c mice were centrifuged and stored at −80 °C until analysis. Nitrite was measured in blood serum samples by enzyme linked immunosorbent assay (ELISA). Samples (50 μl) were added to a 96 wells plate in addition with 50 μl of Griess solution and the absorbance was measured in a TP reader (Thermo Plate) in 550 nm with the obtained values adjusted to the standard curve.

2.4. Histological analysis

Lungs were fixed in 4% buffered formalin followed by paraffin embedded, cut into 4 μm sections that were placed on glass slides, stained with hematoxylin and eosin (H&E), and these sections were reviewed by a pathologist (blinded to the treatment arm).

2.5. Measurement of nodule area

The areas of lung adenomas (H&E staining slides) were measured with the help of an image analysis system. For this purpose, each lesion was photographed, saved in the software Image Pro Plus 3.0, drawn round with an electronic pen and its area was immediately calculated by UHTSCSA Image Tool 3.0.

2.6. Statistical analysis

Data are expressed as mean and standard deviation and the statistical analysis were performed by ANOVA followed by Tukey's pairwise comparisons. The level of statistical significance was defined as $p\text{-value} < 0.05$.

3. Results

In Urethane group followed during 120 days we observed 90% of mice survival whereas no death occurred in the Control group (data not shown). Fig. 1 shows lung histology with hyperplasia in the Control group and hyperplasia + nodules in Urethane injected mice. In Table 1 we observe that spontaneous hyperplasia developed in one animal from the Control group whereas 100% mice from the Urethane group presented hyperplasia and/or nodules. The lungs from the Urethane-injected mice presented nodules with area range from 0.029 to 5.5 mm^2. Histology was considered normal in the lung of mice injected with Urethane and evaluated 30 or 20 days later (data not shown).

During tumor development effector cells from the immune system play in important anti-cancer role. However, this effector response can be impaired by other cells such as T regulatory cells or myeloid suppressor cells. Therefore, our next step was to investigate the immunologic events at early time points after the Urethane-induced lung cancer.

Cell numbers were higher in spleens from the 20 d group in comparison with the 30 d and Control groups (Fig. 2A). Lung cell

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