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Ehrlich ascites tumour unbalances splenic cell populations and reduces responsiveness of T cells to *Staphylococcus aureus* enterotoxin B stimulation

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Abstract

Tumours must avoid host immune response to survive and proliferate; to achieve this purpose, tumours interact with cells of the immune system by means of tumour secreted factors. The alterations of splenic cell populations in mice bearing the Ehrlich ascites tumour have been studied. A rapid and acute response was observed, characterized by a decrease in both CD4 and CD8 T cells, and a transient increase in the number of B cells, which peaked 2 days after tumour inoculation. An increase in macrophage population and in the homing antigen CD18 was also detected. In vitro incubations of splenic cells with the *Staphylococcus aureus* enterotoxin B (SEB) showed that tumour induces a state of reduced responsiveness to stimulation of T cells, mainly affecting CD8 T cells, and a diminished IFN-γ expression.

Keywords: Ehrlich ascites tumour; Spleen; Immune response; *Staphyloccocus aureus* enterotoxin B

1. Introduction

Ehrlich ascites tumour is a rapidly growing carcinoma with very aggressive behaviour. It is characterized by a high rate of glutamine consumption [1], by secreting a variety of immunosuppressive cytokines [2,3] and by a lack of class II MHC expression [4]. These events result in a dysfunction of T and NK cell immunosurveillance [5–7] and in the recruitment of suppressive macrophages [5]. Notwithstanding, Ehrlich ascites cells are clearly immunogenic since mice inoculated with attenuated tumour cells are capable of actively recognizing them as foreign, and acquire a memory that will reject a second inoculation of live Ehrlich ascites tumour cells [8].

In recent studies on Ehrlich ascites tumour model [9], our group has found early tumour effects on Th lymphocytes of the host, accounting for a dramatic decrease in the number of Th cells in the spleen of tumour-bearing mice 2 days after tumour inoculation. This decrease is accompanied with a reduction in the number of CD4 cells expressing IFN-γ after in vitro polyclonal stimulation with PMA and ionomycin.

Since host immune responses to tumour antigens are weak and not immediate [10], the early contacts between host and tumour are critical in determining the future directions of the immune response elicited by tumour antigens. We have recently demonstrated the presence of TGF-β1 in the tumour cells and ascites of tumour-bearing mice [9]. The lack of functional receptors [11] allows tumour cells to avoid the potent growth inhibitory effects of TGF-β on itself and to send an inhibitory signal to the immune system [12]. In this work, we intended to extend the study of the tumour-induced changes in the number of the different splenic cell populations. Furthermore, the responsive status of T cells during the first days of tumour growth was undertaken using SEB. This closely resembles the physiologic activation pathway of T cells, and allowed us to assess the presence of tumour signals that inhibit the immune system response.

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2. Materials and methods

2.1. Animals and tumour cells

A hyperdiploid Lettré strain of Ehrlich ascites tumour cells was maintained in female albino Swiss CD1 mice (24–25 g), purchased from Criffa (Barcelona, Spain), as described elsewhere [1]. The life span of animals after inoculation with 5×10^6 tumour cells was 16 ± 1 days. In this work, tumour-bearing mice were euthanized on day 10 after tumour inoculation.

2.2. Antibodies

The following anti-mouse antibodies were used: IFN-γ, clone XMG 1.2, FITC-labelled from Pharmingen (San Diego, CA); IL-4, clone BVD-24G2, FITC-labelled, from Caltag Laboratories (Burlingame, CA); CD4, clone RM4 -5, CD8a, clone 53-6.7, CD18, clone C71/16, and CD45R clone RA3-6B2, PE-labelled from Sigma (St Louis, MO); anti-mouse macrophage clone F4/80, PE-labelled, from Caltag Laboratories.

2.3. In vitro stimulation of spleen cells

Mice were sacrificed by cervical dislocation. Spleens were homogenized and cells incubated in RPMI medium containing 0.3 mg/ml glutamine, 10% FCS (BioWhittaker), 100 U/ml penicillin, 0.1 mg/ml streptomycin and 1.25 U/ml amphotericin B (Gibco BRL), in 100-mm diameter Petri dishes at 2×10^6 cells/ml. Cells were stimulated for 6 days with 5 μg/ml SEB. After 3 days, cells were supplemented with fresh medium. Cells were harvested, washed with PBS and restimulated for 5 h with 5 ng/ml PMA and 1 mM ionomycin. Brefeldin A (Sigma) was used at 10 μg/ml (from a stock of 1 mg/ml in ethanol) 2 h before the cells were harvested, to prevent secretion of cytokines into the medium [13]. Afterwards, the cells were washed and resuspended in PBS at 2×10^6 cells/ml. Cells were fixed for 20 min at room temperature by addition of 1 vol. of 4% formaldehyde in PBS. Fixed cells were washed twice in PBS and resuspended in PBS supplemented with 0.5% BSA and 0.02% NaN_3 (PBSAN) at $1–2 \times 10^6$ cells/ml. Cells were stored at 4°C in the dark until processing.

2.4. Cytokine staining

Spleen cells were analysed for production of cytokines by intracellular staining as described previously [14]. Briefly, $1–2 \times 10^6$ cells were incubated in 30 μl with the FITC-conjugated anti-cytokine antibody for 15 min at room temperature in PBSAN buffer containing 0.5% saponin, and washed once with the same buffer. For subsequent surface staining, intracellularly stained cells were resuspended in PBSAN buffer and incubated again in 30 μl with the appropriate antibody for 30 min on ice, washed and resuspended in 1 ml PBSAN buffer for analysis.

All antibody incubations were supplemented with 10% normal mouse serum (Sigma) to prevent unspecific binding. Samples were analysed by flow cytometry in FACSort equipment with CellQuest software (Beckton Dickinson).

3. Results and discussion

3.1. Time course of splenic cell populations during the exponential phase of tumour growth

As mentioned above, Ehrlich ascites tumour is a rapidly growing carcinoma. Mice inoculated with 5×10^6 tumour cells have a life span of 16 ± 1 days [1]. Exponential tumour growth was observed up to 10 days after tumour inoculation [15], and dramatic changes in the energy and nitrogen metabolism take place in the host tissues and organs [16,17]. We planned an experimental schedule aimed at the description of the different splenic cell populations during the first 7 days of tumour growth. Mice were inoculated with 5×10^6 Ehrlich ascites tumour cells in the middle of the exponential growth phase (day 7 after transplantation) and sacrificed on days 2, 4 and 7. After homogenization, spleen cells were washed in PBS and fixed in 2% formaldehyde. Stained cells with the appropriate surface antibody were analysed by flow cytometry. The results are shown in Fig. 1 as a total number of different cell types in the spleen. Spleen size, expressed as the total number of cells, increased progressively as the tumour grew (Fig. 1a). The number of T cells (CD4 and CD8) rapidly decreased with tumour growth (Fig. 1b,c). On the contrary, other splenic populations like macrophages and CD18 cells (Fig. 1d,f, respectively) significantly increased. These results reflect that new activated macrophages are being generated, since the antigen that recognizes F4/80 is upregulated through the activation process of macrophages [18]. B cells increased more than twofold on day 2, and then steadily decreased to control values on day 7 (Fig. 1e). CD18 cells followed a pattern similar to that of macrophages, but with a higher number of cells, doubling values of controls on day 7 after tumour inoculation (Fig. 1f).

The balance of cell populations in the spleen is clearly modified by the tumour cells growing in the peritoneum. Previous reports have shown that Ehrlich ascites tumour cells elicit a strong antibody response mediated by IgM. The target antigens were mucine proteins shed by the tumour [19]. The recruitment of B cells to the spleen of tumour-bearing mice in the first days of tumour growth could be explained by the

development of the antibody response directed against tumour antigens.

The CD18 antigen is expressed in all bone marrow derived cells; it is part of the integrin family of receptors and is upregulated during the cell activation process, targeting cells to peripheral organs. Thus, the increased percentage of CD18 cells indicate that these cells are being activated and targeted to peripheral sites.

The picture described here reveals that there is, indeed, an effect of the tumour on the cells of the immune system, involving cell migration and unbalancing different cell populations in the spleen. This results in a net decrease of T cells and an increase of macrophages. The lower number of T cells in the spleen of tumour-bearing mice could be related to an inhibitory effect over them. To study possible tumour effects on T cell functionality, a new approach was carried out.

3.2. In vitro SEB stimulation of splenocytes: responsiveness and IFN-γ expression

Superantigens combine with MHC class II molecules to form the ligands that stimulate T cells via the V_β element of the T cell receptor [20]. Primary in vitro stimulation with the superantigen SEB promotes clonal expansion of T cells carrying specific V_β elements. Restimulation with other stimuli, like PMA, induces cytokine secretion following a pattern that reproduces that of the primary conditions during the SEB stimulation [21]. In order to test whether tumour induced changes of splenic T cell numbers are accompanied by functional alterations, an in vitro primary SEB stimulation protocol was set up.

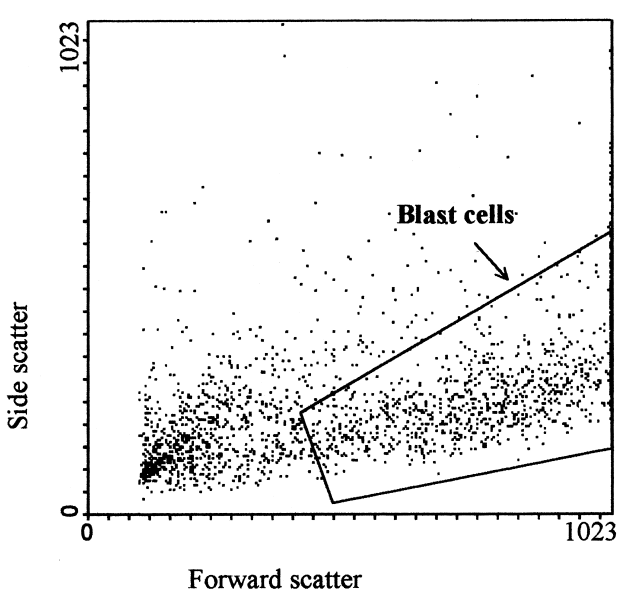

Fig. 1. Time course of different splenic populations in mice bearing Ehrlich ascites tumour cells. Splenocytes were collected, fixed and analysed by flow cytometry at different time points after tumour inoculation. Labelled antibodies (shown as inserts in figures) against cell surface markers were used to characterise different splenic populations. Data are means ± S.E.M. of at least three different mice.

Spleen cells from healthy mice or 2-day tumour-bearing mice were stimulated in vitro with 5 μg/ml SEB and restimulated with PMA and ionomycin, as indicated in Section 2. We used 2-day tumour-bearing mice because this was the earliest time point in which tumour effects on CD4 cells were maximum [9].

The response to SEB stimulation was measured in terms of blast formation. Large activated lymphocytes were selected according to the light scattering parameter during the flow cytometric analysis [22] (Fig. 2). Data in Table 1 show that T lymphocytes from tumour-bearing mice presented significantly fewer activated T cells than control animals, indicating that the presence of the tumour reduces cell responsiveness to stimulation. The inhibitory effect was more pronounced on CD8 cells. While the percentage of blast expressing CD4 decreased by 13% in tumour-bearing mice, CD8 T cells were reduced by 58% (Table 1).

Previous reports have shown that the growth of Ehrlich ascites tumour cells reduce lymphocyte proliferative response to mitogens [7], involving NK as well as T cytotoxic cells [5,7]. Here we use SEB to activate T cells, which resembles the physiological activation pathway more closely than mitogens. We have previously shown that Ehrlich ascites tumour cells produce and release TGF-β to the ascitic fluid [9]. Addition of TGF-β during the stimulation of human T cells with anti-CD3, PMA or PHA, inhibits the proliferation of T cells by down regulating IL-2 proliferative signals [23]. Besides, tumour TGF-β inhibits Th function, and blocks the T help for tumour removal [24]. In fact, the neutralization of TGF-β with antibodies or antisense oligonucleotides has proved to be a useful way to enhance the immune system anti-tumour response, inhibiting tumour growth and enhancing NK cell activity [12,25,26]. Tumour TGF-β could then be a possible vehicle to depress immune system responsiveness to stimulation. In addition, Ehrlich ascites cells have been shown to shed tumour antigens that interact with the host immune system. Among these antigens is the protein MUC-1, that both blocks the antibody response anti-MUC-1 by opsonizing the free antibody and inhibits the activation of T cells [27,28].

The diminished proliferative response to SEB of T cells from tumour-bearing mice is accompanied by a reduction in the number of IFN-γ expressing cells (Table 2). Samples were analysed for IL-4 expression but no positive cells were found (results not shown). The decrease of IFN-γ expression agrees with the hypothesis that tumour inhibits the Th1-type response of Th cells, mediated by IFN-γ, which would promote tumour regression [29–31].

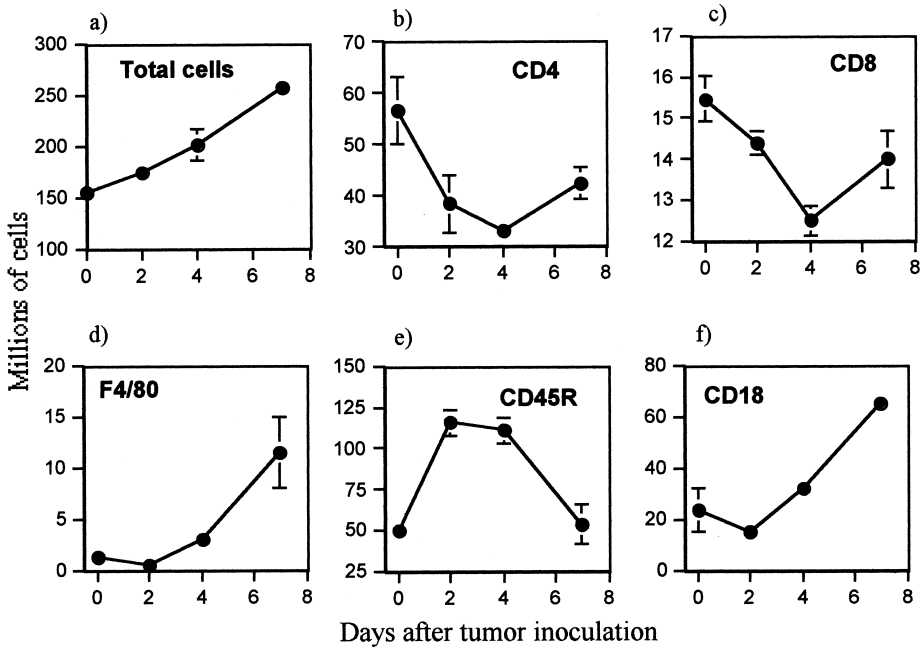

Fig. 2. Blast cell selection for analysis of flow cytometric data. Dot plot representation of 6-day SEB stimulated splenocytes. Selection of blast gate was done in a forward/side light scatter representation of the flow cytometric data as depicted above.

Our work demonstrates that Ehrlich ascites tumour cells induce rapid and drastic changes in splenic cell populations of the tumour-bearing mice, and a state of T-cell unresponsiveness to stimulation via the T-cell receptor-MHC class II interaction. The V_β elements of the T-cell receptor that recognize SEB are broadly represented in the mouse immune system, implying that the tumour induces systemic effects on T cells. The understanding of the mechanisms that the tumour uses for systemic T-cell inhibition would be of great value to design improved anti tumour therapies.

Table 1
Percentages of T lymphocytes blast after SEB stimulation[a]

Treatment	% Total T-cell blast	% CD4 blast	% CD8 blast
Control	15.6 ± 0.7	10.1 ± 0.4	5.5 ± 0.4
Tumour-bearing mice (day 2)	11.2 ± 0.3*	8.8 ± 0.5*	2.4 ± 0.3*

[a] Splenocytes from healthy (control) or 2-day tumour-bearing mice were cultured in vitro with SEB for 6 days and then restimulated for 5 h with PMA and ionomycin. Figures are means of at least three animals ± S.E.M.

* $P \leq 0.05$ when compared with control values as assessed by Mann–Whitney *U*-test.

Table 2
IFN-γ expression on T cells from SEB stimulated splenocytes[a]

	IFN-γ expressing	
Treatment	CD4 blast	CD8 blast
Control	21.1 ± 5	30.6 ± 4.8
Tumour-bearing mice (day 2)	12.3 ± 3.1*	20.2 ± 2.8*

[a] Splenocytes were culture as described in Table 1. Figures are means of at least three animals ± S.E.M.

* $P \leq 0.05$ when compared with control values as assessed by Mann–Whitney *U*-test.

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