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REGULATORY B CELLS PRESENT IN LYMPH NODES DRAINING A MURINE TUMOR

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Abstract In cancer, B cells have been classically associated with antibody secretion, antigen presentation and T cell activation. However, a possible role for B lymphocytes in impairing antitumor response and collaborating with tumor growth has been brought into focus. Recent reports have described the capacity of B cells to negatively affect immune responses in autoimmune diseases. The highly immunogenic mouse tumor MCC loses its immunogenicity and induces systemic immune suppression and tolerance as it grows. We have previously demonstrated that MCC growth induces a distinct and progressive increase in B cell number and proportion in the tumor draining lymph nodes (TDLN), as well as a less prominent increase in T regulatory cells. The aim of this research was to study B cell characteristics and function in the lymph node draining MCC tumor and to analyze whether these cells may be playing a role in suppressing antitumor response and favoring tumor progression. Results indicate that B cells from TDLN expressed increased CD86 and MHCII co-stimulatory molecules indicating activated phenotype, as well as intracellular IL-10, FASL and Granzyme B, molecules with regulatory immunosuppressive properties. Additionally, B cells showed high inhibitory upon T cell proliferation ex vivo, and a mild capacity to secrete antibodies. Our conclusion is that even when evidence of B cell-mediated activity of the immune response is present, B cells from TDLN exhibit regulatory phenotype and inhibitory activity, probably contributing to the state of immunological tolerance characteristic of the advanced tumor condition.

Key words: tumor immunity, B regulatory cells, tolerance

Resumen ***Presencia de células B con propiedades regulatorias en ganglios drenantes de un tumor murino.*** En cáncer, las células B han sido clásicamente asociadas a la presentación antigénica, secreción de anticuerpos y activación de células T. Recientemente se comenzó a investigar un posible rol negativo de los linfocitos B sobre la respuesta inmune antitumoral, debido a que se describieron ciertas subpoblaciones B con capacidad de afectar negativamente la respuesta inmune en enfermedades autoinmunes. El tumor murino MCC es altamente inmunogénico en estadios tempranos; a medida que crece pierde su inmunogenicidad e induce inmunosupresión sistémica y tolerancia. En este modelo hemos demostrado previamente que el crecimiento tumoral induce un marcado y progresivo aumento en el número y proporción de células B en los ganglios drenantes del tumor (TDLN) y un aumento menos marcado de células T regulatorias. El objetivo fue estudiar las características y función de las células B en los ganglios que drenan el tumor MCC y analizar si éstas podrían inhibir la respuesta contra el tumor favoreciendo su progresión. Encontramos que las células B en los TDLN presentan expresión aumentada de las moléculas CD86 y MHCII indicando su activación, y expresión intracelular de IL-10, FASL y Granzima B, moléculas con función inmunosupresora. Además, mostraron alta actividad inhibitoria de la proliferación T *ex vivo* y moderada secreción de anticuerpos. Los resultados indican que aun cuando persisten marcadores de activación de la respuesta inmune, las células B presentes en los ganglios drenantes del tumor muestran fenotipo regulatorio y actividad inhibitoria, sugiriendo que las mismas contribuirían al estado de tolerancia inmunológica característico del estadio de tumor avanzado.

Palabras clave: inmunidad tumoral, células B regulatorias, tolerancia

The involvement of the host immune system in the control of cancer progression has been assessed for several years; nowadays, it is vastly accepted that anti-tumor immunity occurs and that tumors have evolved an elaborate assembly of tricks to avoid immune- mediated rejection[1]. The mechanisms responsible of immune cell dysfunction in patients with cancer involve a wide diversity of soluble immunosuppressive factors (such as TGFβ, IL-10 and the inhibitory ligands FasL and TRAIL) released by tumor cells or by various suppressor cells in the tumor microenvironment, including regulatory T cells (Tregs). In the last decades Tregs have been the major target

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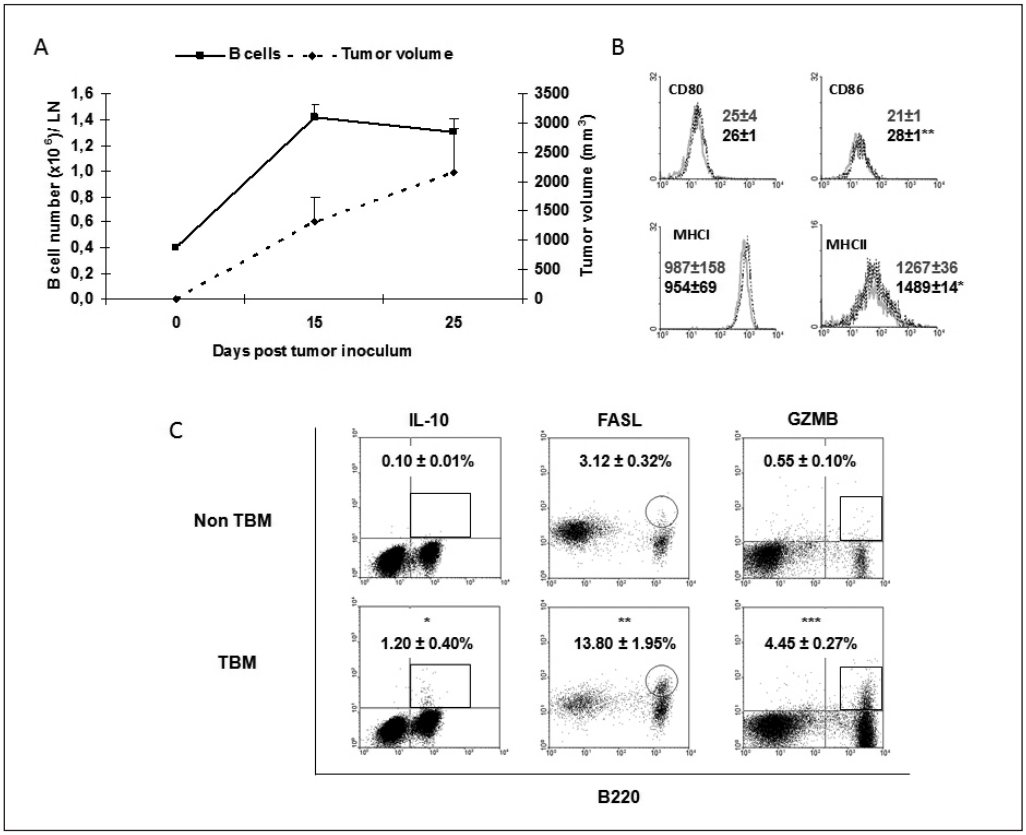

Fig. 1.– Phenotype of B cells present in lymph nodes draining the tumor (TDLN). Lymph nodes from control and tumor bearing mice were aseptically disaggregated and 1x10^6 cells were incubated with fluorochrome-conjugated monoclonal antibodies. For intracellular staining cells were fixed and permeabilized prior to antibody staining. Cells were analyzed using a FACS flow cytometer and Winmdi software. One of three comparable experiments is shown (n = 3 to 4 mice per group, *p < 0.05, **p < 0.01, ***p < 0.001). **A)** B cell number increases as tumor grows. B cells were identified by anti-B220 and counted by flow cytometry. A representative tumor growth curve is included. **B-C)** TDLN B cells exhibit predominantly regulatory phenotype. Surface expression of MHCI, MHCII, CD80 and CD86 (black for tumor bearing mice and grey for non tumor bearing mice) and FasL and intracellular expression of IL-10 and GZM B were assessed at day 15.

of efforts to therapeutically modulate their inhibitory activity in order to achieve tumor remission or prevent recurrences[2, 3]. More recent studies are currently focusing on the role of B cells in regulating the immune response. Although these cells have been classically associated with antibody secretion, antigen presentation and antitumor-T cell activation, a possible role for B lymphocytes in impairing antitumor response and collaborating with tumor growth has been brought into focus due to the recently described participation of a regulatory subset of B cells in autoimmune diseases[4].

The mouse sarcoma MCC is a highly immunogenic tumor widely used to study the effect of tumor development on the immune system[5, 6]. As MCC grows, its immunogenicity declines and a state of tolerance against the tumor arises[7, 8]. We have recently demonstrated that MCC growth is accompanied by a marked and progressive increase in B cell number and proportion in the tumor draining lymph nodes (TDLN), along with a less prominent increase in Tregs[7]. While the participation of Tregs in the inhibition of the antitumor immune response has been broadly assessed[9] the role of B cells is still a matter of debate. The aim of this paper was to study the characteristics of the B cell population present in the tumor draining lymph nodes, and to analyze whether these cells may play a role in favoring the establishment of tolerance and tumor progression.

As we previously showed[7], MCC growth increased B cell number at the TDLN during the immunogenic phase, with a peak at day 15 after tumor inoculum (Fig. 1a). We therefore obtained cells from TDLN of tumor bearing mice (TBM) on day 15 and analyzed B cell phenotype and function. As control, non tumor bearing mice (non- TBM) B cells were obtained from inguinal and axillary LN. We found that B cells from TBM exhibited activated phenotype characterized by increased CD86 and MHCII expression

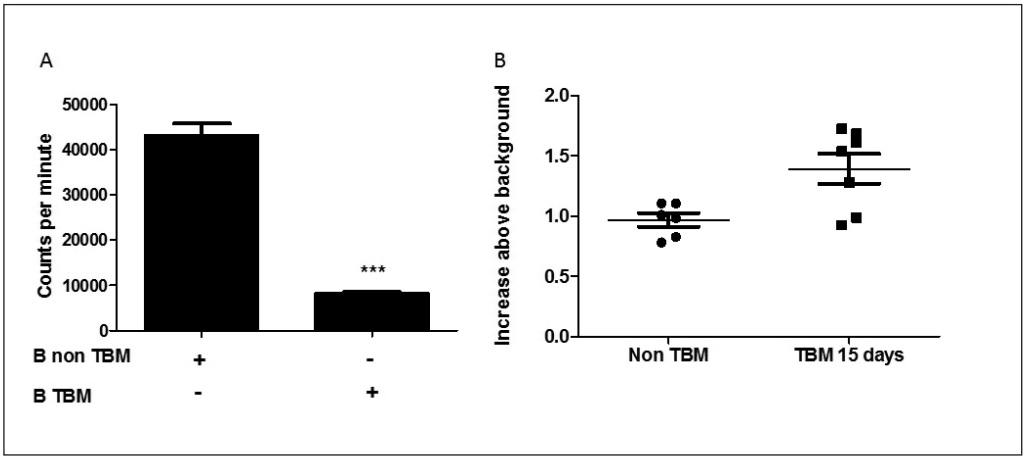

Fig. 2.– Function of B cells present in TDLN. **A)** B cells from tumor bearing mice suppress T cell proliferation. Mitomycin C –treated AKR mouse spleen cells ($2x10^5$) were cultured with Balb/c LN cells ($2x10^5$) to generate an allogeneic stimulus for 96 h, with a final 18 h pulse of 1 µCi/well [^{3}H]-thymidine. Incorporated radioactivity was measured in a liquid scintillation Beta counter. Mitomycin C- treated B cells ($75x10^3$) isolated from Balb/c control or tumor bearing mice were added from the beginning of the culture. One of two reproducible experiments is shown (n = 3 mice per group, $p < 0.001$). **B)** Tumor-reactive antibodies are present in sera from tumor bearing mice. Binding of sera IgG to tumor cells was evaluated by indirect ELISA, after incubating sera and MCC cells with anti-mouse IgG coupled to HRP and TMB as substrate (one of two experiments done n = 6 - 7, $p < 0.05$).

(Fig. 1b). Interestingly, these cells also expressed IL-10, FASL and Granzyme B (Fig. 1c), which were shown to induce immunosuppression in other systems[10]. On the other hand, B cells isolated from TBM showed inhibitory effect upon allogenically-induced T cell proliferation (Fig. 2a), while no effect was observed on MCC cultures (data not shown). Finally, a moderate titer of specific anti- MCC antibodies was detected in the serum of TBM (Fig. 2b), indicating that B cells retain their ability as antibody secreting-cells.

Discussion

Immune antitumor response emerges from the balance between regulatory and activated cells. It is now accepted that tumor presence leads to the appearance of regulatory immunosuppressive cell populations that not only affect endogenous antitumor response but also weaken the efficacy of immunotherapies. Regulatory T cells have been vastly assessed as one of the main inhibitory cell population. However, multiple regulatory mechanisms have been proposed in the last years for B cells in different abnormal immune system conditions. Some of them need cell to cell contact to be effective (FasL and PDL2) while others are mediated by B cell- secreted soluble molecules, TGFβ, Granzyme B and IL-10[10].

Together with signs of immune activation, such as increased expression of the surface markers CD86 and MHCII and secretion of antibodies against MCC, B cells from lymph nodes draining the tumor seemed to be predominantly immunosuppressive, as suggested by the expression of IL-10, FasL and Granzyme B. Importantly, these cells were able to inhibit in vitro- induced T cell proliferation, which could indicate that the negative effect of B cells on tumor immunity is mostly indirect through affecting T cells.

The presence of regulatory B cells was recently proposed to favor tumor progression in other models. Interleukin-10 secreted by B cells- was shown to reduce IFNγ secretion by cytotoxic CD8+ T cells[11] and increase Treg cells presence[12], thus impairing antitumor reaction. On the other hand, Granzyme B expressed by B cells infiltrating tumors had the capacity to negatively regulate T cell proliferation[13].

Results presented herein indicate that along with some evidences of B cell- mediated activation of the immune response, the tumor also induces the emergence of a population of B cells with regulatory characteristics. We propose that these cells through different mechanisms such as IL-10, Granzyme B and FasL expression, could impede a proper antitumor response and collaborate with the immunological tolerance detected in advanced tumor bearing hosts.

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Conflict of interest: None to declare.

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LA TAPA

G. Guidi. **Hemofagocitosis**. Grabado basado en el diseño de Guidi.

Ilustración tomada de Guido Banti (1852-1925) *Anatomia Patologica. Volume primo. Parte II. Capitolo ottavo. Infezione tifiche* (p116-30), p 122. Milano: Società Editrice Libraria, 1907.

La leyenda dice: “Fig. 42. Bazo en la fiebre tifoidea: *a* residuo de un folículo, con pocos linfocitos y algunas células epitelioides; *b* porción de pulpa remanente infiltrada de glóbulos rojos; *c* macrófago con linfocitos en variados períodos de destrucción; *d* macrófago conteniendo glóbulos rojo, con núcleo normal; y *e* y *h* macrófagos necróticos, sin núcleo reconocible, conteniendo glóbulos rojos hinchados; *f* masa homogénea proveniente de la fusión de células necróticas; *g* fragmentos de células necróticas (ocular 3, objetivo 8, Koritska)”. En el texto: “En la pulpa se ven numerosos macrófagos, similares en aspecto a aquellos descriptos en el intestino y glándulas linfáticas las cuales fagocitan y destruyen con mucha actividad linfocitos y glóbulos rojos”.

Los macrófagos que fagocitan células sanguíneas (hemofagocitosis) se llamaron células de Rindfleisch, por Georg Eduard von Ridfleisch (1836-1908) que las denominó “células tíficas”; se encuentran en el tejido linfoide del intestino, ganglios linfáticos y bazo. Las discusiones acerca de su origen fueron violentas. Para William George MacCallum (1874-1944) las describieron, y notaron su capacidad fagocítica, Friedrich Albin Hoffmann (1843-1924) y Christian Albert Theodor Billroth (1829-1894). Frank Burr Mallory (1862-1941) decidió que eran células endoteliales. Felix Jacob Marchand (1846-1928) que derivaban de las células reticulares y endoteliales, y Sergius Saltikow (¿18xx?-19xx?) que eran células endoteliales de senos linfáticos mezcladas con células linfoides de varios tipos.

MacCallum dice que no son peculiares de la fiebre tifoidea e idénticas a las observadas en la tuberculosis y otras afecciones y prefiere considerarlas macrófagos, miembros de la familia de las células migrantes mononucleares. El tiempo dio la razón a MacCallum quien no menciona a Rindfleisch. (MacCallum WG. A Textbook of pathology. Philadelphia: Saunders, 1926, 4th. Ed. Chapter XXI, Typhoid infections, p560-577). Ver también: Fisman DN. Hemophagocytic Syndromes and Infection. *Emerging Infec Dis* 2000; 6: 601-8; Zoller EE, Lykens JE, Terrell CE, et al. Hemophagocytosis causes a consumptive anemia of inflammation. *J Exp Med* 2011; 208:1203-14; Usmani GN, Woda BA, Newburger PE. Advances in understanding the pathogenesis of HLA [Haemophagocytic Lymphohistiocytosis] *Br J Haematol* 2013; 16: 609-22.