Immunosurveillance of Erbb2 Carcinogenesis in Transgenic Mice Is Concealed by a Dominant Regulatory T-Cell Self-Tolerance

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Abstract
To assess the role of CD4+CD25+Foxp3+ regulatory T (Treg) cells in overcoming immunosurveillance of Erbb2 (HER-2/neu) mammary lesions, we studied the effects of their sustained removal in BALB/c female mice made transgenic for the rat Erbb2 (r-Erbb2) oncogene (BALB-neuT mice), which develop multiple mammary carcinomas. During the progression of these lesions, Treg cells expand in the spleen, tumor draining lymph nodes, and tumors. Repeated administration of anti-CD25 antibodies extends tumor-free survival, reduces carcinoma multiplicity, and leads to the manifestation of a natural antibody and CTL-mediated reactivity against r-Erbb2. Loss of Foxp3+ Treg cells during anti-CD25 treatment remarkably caused the disappearance of Gr1+ immature myeloid cells, suggesting a cross-talk between these two inhibitory immune cell types. Treg cell expansion associated with r-Erbb2 overexpression may be seen as a physiologic response to dampen the immune reaction elicited by local anomalous overexpression of a self-antigen. (Cancer Res 2006; 66(15): 7734-40)

Introduction
Overexpression of the Erbb2 (HER-2/neu) oncogene by a significant percentage of human carcinomas is associated with aggressive tumor growth, greater invasiveness, enhanced metastatic potential, and increased resistance to therapy (1). The protein product coded by Erbb2, p185, is a member of the epidermal growth factor receptor family endowed with a potent tyrosine kinase activity that plays crucial roles in physiologic processes, such as embryogenesis, cell proliferation, and apoptosis (2). However, as p185 is a self-antigen poorly expressed by the cells of adult individuals, patients with clinically evident carcinomas overexpressing Erbb2 may display a natural antibody and cell-mediated response to p185 (3, 4). These are too small and too late to enhance resistance to tumor expansion. Their induction, however, suggests that p185 overexpression triggers an immune response. This is an important issue, because elicitation of an immune response to p185 is the result of immune tolerance to self-antigens. Clonal deletion of T and B cells recognizing p185 with high avidity irreversibly impairs the immune repertoire. Lower-affinity responses and responses to subdominant epitopes of self p185 that were not deleted may be inhibited by regulatory T (Treg) cells (7), interleukin (IL)-13–producing natural killer (NK) T cells (8), and immature myeloid cells (9) as well as additional mechanisms providing a negative regulation of the immune response of autoreactive T cells.

To study the surmounting of natural immunosurveillance during Erbb2 carcinogenesis, we used female BALB/c mice made transgenic for the rat Erbb2 (r-Erbb2) transforming oncogene (BALB-neuT). All these female mice develop a multifocal carcinoma in each of their 10 mammary glands (10) with a stepwise progression that mimics a few typical features of human Erbb2 carcinogenesis (11). This progression can be exploited to study the natural expansion of CD4+CD25+ Treg cells expressing the Foxp3 transcription factor and the glucocorticoid-inducible tumor necrosis factor receptor (GITR; ref. 12).

Transgenic Erbb2 is the only genetic difference between BALB-neuT and wild-type BALB/c mice. They can thus be compared with assess the consequences of progressive overexpression of a self-antigen in the expansion of Treg cells, because these are physiologically involved in inhibiting the immune response and maintaining homeostatic tolerance to self-antigens (12, 13). As Treg cell removal with antibodies or defects in their maturation may result in various forms of autoimmunity (12–15), reduction of their expansion may disclose the presence of a natural immunosurveillance to overexpressed rat p185 (r-p185) in BALB-neuT mice and may pose the basis for the design of more effective immunologic maneuvers in tumor prevention and treatment.

The results of the present study show that Treg cells expand in BALB-neuT mice during the lengthy progression of Erbb2-driven mammary carcinogenesis. Their sustained removal discloses an antibody and CTL-mediated natural immunosurveillance able to hamper the progression of autochthonous Erbb2 lesions.

Materials and Methods
Mice. Severe combined immunodeficient (SCID) and BALB/c (H-2d; 6-8 weeks old) female mice were obtained from Charles River Italia SpA (Calco, Italy). Mammary cancer-prone BALB-neuT female mice (H-2d) overexpressing the r-Erbb2 transforming oncogene under the control of the mouse mammary tumor virus promoter (10) were bred for us under specific pathogen-free conditions at Charles River Italia. These mice were randomly assigned to control and treatment groups and concurrently treated. Mammary glands were inspected weekly to note tumor appearance. Progressively growing masses >1 mm mean diameter were regarded as tumors. Tumor multiplicity was calculated as the cumulative number of...
incident tumors divided by total number of mice and is reported as mean ± SE (10). Each neoplastic mass was measured with calipers in two perpendicular diameters and its volume was calculated as (L² × W) / 2, where L and W represent the long and short diameters, respectively. Total tumor volume is the sum of individual tumor volumes of each mouse and is reported as mean ± SD. In all operations, mice were treated in accordance with the European Community guidelines for the protection of animals used in scientific procedures.

Production and administration of anti-CD25 antibodies. The PC61 hybridoma-secreting IgG1 mAbs to the α-chain of murine IL-2 receptor (CD25; ref. 16) was purchased from the American Type Culture Collection (11, 12). To evaluate the presence of Foxp3+ cells in mammary glands, previously in detail on groups of five BALB-neuT mice of progressive age (11, 12), we used a magnetic cell separation technique. Rats were sacrificed at ages 7, 13, 19, and 25 weeks. For in vitro assays, Spc were restimulated in vitro by mitomycin C-treated 3T3-NKB cells in the presence of 10 units/mL recombinant IL-2 (Eurocetus, Milan, Italy). In some experiments, Spc were restimulated by adding 2.5 μg/mL of the r-p185 63-71 nonamer peptide (TYPVANASL; ref. 18; InBios SrL, Biotech Products, Naples, Italy) to bind the H-2d glycoproteins with high affinity (http://www.syfpeithi.de/). Restimulated Spc were assayed in a 4-hour Na2CrO4 (50 μM, Perkin-Elmer, Boston, MA) release assay at E/T ratios from 50:1 to 61:1 in round-bottomed, 96-well microtiter plates in triplicate as described previously in detail (19).

In vivo cytotoxicity assay was done as described by Ritchie et al. (20), with slight modifications. Briefly, a single-cell suspension of 10⁶ naïve Spc/mL was labelled with 0.5 or 5.0 μmol/L of the fluorescent dye CFSE (Molecular Probes, Leiden, the Netherlands). Spc labelled with 5 μmol/L CFSE (CFSElow) were also pulsed with r-p185 63-71 nonamer peptide for 1 hour at room temperature. The two Spc populations were mixed together in equal amounts and injected i.v. into control and treated mice. Mice were sacrificed 48 hours later, and single-cell suspensions from spleens were processed individually to evaluate the presence of CFSEhigh and CFSElow cells with the CyAn ADP after adding propidium iodide to exclude dead cells. The specific cytolytic activity was calculated as 100 × (percentage CFSElow cells – percentage CFSEhigh cells)/ percentage CFSElow cells.

Statistics. Differences in tumor incidence were evaluated with the Mantel-Haenszel log-rank test, those in tumor multiplicity, number of positive cells at flow cytometry and antibody titer with Student’s two-tailed t test.

Results

Treg cells expand during ErbB2 carcinogenesis. First, we determined whether the progression of carcinogenesis in the mammary glands of BALB-neuT mice triggers the expansion of CD4+CD25+Foxp3+ Treg cells. The atypical mammary hyperplasia generated by cells overexpressing r-p185 first evident at age 4 weeks (11) progresses to multifocal preneoplastic lesions around week 7 (Fig. 1C and H). Multiple carcinomas of ~200 mm³ are palpable in every mouse by week 25 (Fig. 1A, F, and K). At the seventh week, the initial ErbB2 overexpression does not lead to detectable Treg cell accumulation in the spleen (Fig. 1A) and the axillary lymph nodes draining the mammary pad (data not shown) or in the mammary lesions, where Foxp3+ lymphoid cells remain 1 ± 2 per ×400 microscopic field (Fig. 2A). However, the subsequent progression of mammary carcinogenesis is accompanied by an increment in CD4+CD25+Foxp3+ Treg cells in the spleen (Fig. 1A) and the mammary tumors (Fig. 2B). We have shown previously that this progression of BALB-neuT carcinogenesis is also accompanied by an expansion of CD11b+Gr1+ immature myeloid cells (21).

Sustained depletion of CD4+CD25+Foxp3+GITR+ Treg cells through chronic infusion of anti-CD25 IgG. Cancer-prone BALB-neuT mice received repeated infusions of normal rBG or anti-CD25 IgG from the 6th week to the 24th week of age to assess their effect on Treg cells. A dramatic decrease in Treg cells was already evident at week 7 in both the spleen (Fig. 3A) and axillary lymph nodes (Fig. 3B) following the first two administrations of 500 μg anti-CD25 IgG. At week 25, the percentage of Foxp3+GITR+ T cells among total CD4+ cells almost doubled in the spleen and...
markedly increased in the lymph nodes of normal rlgG-treated BALB-neuT mice. By contrast, repeated injections of anti-CD25 IgG, twice in week 6 and then once weekly until week 24, keeps low their percentage in both spleen and lymph node as shown by flow cytometry (Fig. 3A and B). This reduction is mirrored by immunocytochemistry observations (Fig. 3C versus Fig. 3D).

**Treg cell depletion accompanies delayed carcinogenesis.** To determine whether chronic removal of Treg cells unveils an immune response able to hamper the progression of Erbb2 lesions, tumor incidences were compared in normal rlgG-treated and anti-CD25 IgG-treated BALB-neuT mice. Both a marked delay in the appearance of the first tumor and a reduction in the number of palpable tumors per mouse (tumor multiplicity; ref. 11) were evident in anti-CD25 IgG-treated mice. At week 20, when all control mice displayed one or more tumors, 80% of mice treated with anti-CD25 IgG were free of palpable tumors (Fig. 4A). The tumor-free survival curve of anti-CD25 IgG-treated mice was significantly delayed \( (P < 0.0001) \) compared with that of normal rlgG-treated mice. The tumor multiplicity was also significantly lower \( (P < 0.0004 \) to \( P < 0.00004 \)) from weeks 19 to 32 (Fig. 4B). Moreover, at 38 weeks, when the experiment ended, a few mammary glands of BALB-neuT mice receiving anti-CD25 IgG until week 24 did not display a palpable tumor. Depletion of Treg cells through anti-CD25 IgG also leads to a reduced number of CD11b+Gr1+ immature myeloid cells at week 7 and their drastically hindered expansion at week 25 (Fig. 5).

**Treg cell depletion unveils a natural immune response to r-p185.** We next determined whether Treg cell expansion conceals an immune response that may be naturally triggered by r-p185 overexpression. A single weekly infusion of anti-CD25 IgG, but not normal rlgG, uncovers both a significant antibody (Fig. 6) and a CTL response to r-p185 (Fig. 7). The presence of anti-r-p185 natural antibodies was assayed in the sera from untreated cancer-prone BALB-neuT mice whose each mammary gland was examined as described in Materials and Methods.

- **Figure 1.** Kinetics of tumor progression and CD4+CD25+Foxp3+ Treg cell expansion. A, percentage of CD25+Foxp3+ cells among total CD4+ Spc (left axis, dots) and total tumor volume (right axis, line) evaluated at 6-week intervals in untreated cancer-prone BALB-neuT mice. At each time point, the percentage of CD25+Foxp3+ cells was evaluated individually in groups of 5 mice. There were 25 mice in the experiment at the beginning, and for each determination of CD25+Foxp3+ cells, 5 mice were sacrificed after total volume evaluation. Total tumor volume is the mean ± SD of 25-5 mice. Whole mounts \((B-F)\) and histologic sections \((G-K)\) of mammary glands from BALB/c and BALB-neuT mice. \( B \) and \( G \), saccular terminal end buds \((B)\) and normal duct lined by a single layer of ductal epithelial cells \((G)\) of normal 7-week-old BALB/c mice. In BALB-neuT mice age 7 weeks \((C-H)\), ducts are associated with little dark side buds \((C)\) that histologically appear as ductal protrusions \((H)\). At week 13, these structures surround the ducts \((D)\) and give rise to lobules of atypical hyperplasia and foci of in situ carcinoma \((I)\) that expand and converge into lobular carcinoma \((E)\) and \((J)\). At week 25, large masses \((F)\) of carcinoma with a nodular appearance and divided by solid septa often display central necrosis \((K)\). At week 31, macroscopic tumors are evident in all the 10 mammary glands \((L)\). Magnification, \( \times 20 \) \((B\) and \(C)\), \( \times 16 \) \((D-F)\), and \( \times 200 \) \((G-K)\). Representative images from groups of five mice whose each mammary gland was examined as described in Materials and Methods.

- **Figure 2.** Accumulation of Foxp3+ cells in the mammary lesions. Immunohistochemical staining of Foxp3+ cells (red) on mammary tissue obtained from BALB-neuT mice at week 7 \((A)\) and week 25 \((B)\). A, a single positive cell \((arrow)\) is close to a hyperplastic mammary duct \((>400)\); \(B\), several positive cells are infiltrating the solid tumor mass \((>100)\). Representative images from groups of five mice whose each mammary gland was examined as described in Materials and Methods.
Discussion

Enhancement of immunosurveillance through Treg cell removal has been mostly reported in transplantable tumor models (26–30) but also with tumors growing in transgenic mice (17, 30) and chemically induced tumor development (31). Here, we show that during Erbb2-driven mammary carcinogenesis CD4+CD25+Foxp3+GITR+ Treg cells expand in the spleen, tumor draining lymph nodes, and mammary lesions. The progression of BALB-neuT carcinogenesis is also accompanied by an expansion of CD11b+Gr1+ immature myeloid cells in both the blood (21) and the spleen. Chronic infusion of anti-CD25 IgG into BALB-neuT mice does not simply result in the inactivation of CD4+CD25+ cells (32) but also leads to a sustained physical depletion of CD4+CD25+Foxp3+GITR+ Treg cells. Such removal unveils a natural immunosurveillance against Erbb2-driven autochthonous carcinogenesis.

Because of the mammary overexpression of membrane r-p185, transgenic BALB-neuT mice are genetically predestined to develop multiple invasive and metastasizing mammary carcinomas (10). Many features of their progression, including gene expression profiles, closely mimic what happens in human mammary cancer (11, 33). In these mice, the chronic removal of CD4+CD25+Foxp3+GITR+ Treg cells extends tumor-free survival, reduces carcinoma multiplicity, and leads to the manifestation of a natural antibody and CTL-mediated reactivity against r-p185. It also hinders the expansion of CD11b+Gr1+ immature myeloid cells that goes along with tumor progression (21).

Because the r-Erbb2 transgene is the genetic difference between wild-type BALB/c mice and transgenic BALB-neuT mice, comparison of the immune response in these two lines allows direct assessment of the tolerance to r-p185 as an overexpressed
tumor-associated antigen. BALB/c mice do not express r-p185, which is thus a xenograft antigen differing in several epitopes from mouse p185 (18). Following immunization, BALB/c mice develop a strong immune response to r-p185, and CTL are a significant component of such response (34). The reaction triggered by the vaccine (34) or after Treg cell removal (15) is strong enough to bring about the rejection of large transplanted r-p185 tumor cells. By contrast, in BALB-neuT mice, r-p185 is expressed in the thymus at birth and is progressively increasingly overexpressed by the cells of hyperplastic mammary lesions starting from the fourth week of age (11). Because this r-p185 overexpression, immunoscope analysis of the T-cell repertoire shows that in BALB-neuT mice CTL clones reacting with high affinity with r-p185 peptides are depleted. CD4 T-cell clones able to recognize r-p185 peptides are still present and vaccines elicit an IFN-γ and antibody-mediated immune response that hampers the initial stages of autochthonous carcinogenesis, whereas the CTL response is not evident (19, 22–25, 35).

Very little information is available regarding how tumor-specific Treg cells develop in tumor-bearing hosts. Present data in BALB-neuT mice show that by comparison with age-matched BALB/c mice no major increase in Treg cells is evident during the early stages of mammary hyperplasia. This is not surprising because r-p185 is but one of the innumerable self-antigens against which the autoimmune response is prevented by Treg cells (36). However, as mammary lesions progress and many more cells overexpress r-p185, an expansion of Treg cells becomes evident in the spleen and particularly in the tumors. This late infiltration of Treg cells in ErbB2 carcinomas fits in well with what has been described with transplantable tumors (29) and human cancers (37), where Treg cell accumulation is a late event in well-established tumors. The Treg cell ability to localize to the tumor site seems to permit the close contacts with effector CTL required for interference with their functions. By contrast, Treg cells in the peripheral organs may inhibit CD4 helper function and thus the elicitation of a significant and long-lasting antibody-mediated response (38).

The Treg cell expansion that accompanies r-p185 overexpression may be seen as a physiologic response to dampen the immune reaction elicited by local anomalous overexpression of a self-antigen, a major source of spontaneous autoimmunity (39). However, r-p185 is not only a self-antigen overexpressed on the cell membrane as carcinogenesis progresses but also a signaling receptor that delivers signals triggering the proliferation and survival of normal and tumor cells and whose anomalous overexpression plays a causal role in the promotion of carcinogenesis (1, 2). This double role of r-p185, a self-tolerated antigen playing important physiologic roles and a tumor antigen causally involved in the neoplastic progression, paradigmatically illustrates what may happen with most tumor-associated antigens. These, in fact, are self-antigens and thus display a natural immune recognition and immunosurveillance counterbalanced by a dominant immune tolerance (40, 41).

In several cases, the antigen presented by autochthonous tumors does not promote the dendritic cell activation necessary for proper arousal of effector CD4⁺ and CD8⁺ T-cell responses and results in the induction of tolerance (42, 43). By contrast, present data as well studies in patients (3, 4) suggest that tumor overexpression of p185 is enough to overcome self-tolerance and arouse antibody and cell-mediated immune responses. In the clinical setting, these responses are too small and too late to influence tumor progression. In BALB-neuT mice, they are meaningless as they are buried by Treg cells. Following Treg cell removal, a significant natural surveillance against ErbB2 carcinogenesis is evident. It is, however, only temporarily effective and insufficient to ultimately eradicate the tumor. This failure may rest on the central deletion in BALB-neuT mice of effector T cells recognizing r-p185 with high affinity. Depletion of Treg cells hampers the expansion of CD11b⁺Gr1⁺ immature myeloid cells. In the spleen of BALB-neuT mice treated with 500 μg anti-CD25 IgG twice in week 6 and then once weekly until week 24, Treg cell depletion goes along with CD11b⁺Gr1⁺ immature myeloid cell reduction at week 7 (top right) and with a lack of their expansion that accompanies mammary carcinogenesis (21) at week 25 (bottom right). GR1 and CD11b profiles of total Spc were depicted as dot plots. The number in the top right quadrant represents the percentage of CD11b⁺Gr1⁺ cells within the Spc. Representative dot plot from one of the five mice individually analyzed and that gave homogeneous results.

**Figure 5.** Depletion of Treg cells hampers the expansion of CD11b⁺Gr1⁺ immature myeloid cells. In the spleen of BALB-neuT mice treated with 500 μg anti-CD25 IgG twice in week 6 and then once weekly until week 24, Treg cell depletion goes along with CD11b⁺Gr1⁺ immature myeloid cell reduction at week 7 (top right) and with a lack of their expansion that accompanies mammary carcinogenesis (21) at week 25 (bottom right). GR1 and CD11b profiles of total Spc were depicted as dot plots. The number in the top right quadrant represents the percentage of CD11b⁺Gr1⁺ cells within the Spc. Representative dot plot from one of the five mice individually analyzed and that gave homogeneous results.

**Figure 6.** Depletion of Treg cells unveils a natural antibody response to r-p185. Titers of anti-r-p185 antibodies and median (horizontal line) in the sera of BALB-neuT mice repeatedly infused with normal rIgG (○) or anti-CD25 IgG (●). BALB-neuT mice receiving anti-CD25 IgG (five mice at week 10 and seven mice at week 25) displayed a higher titer than mice receiving normal rIgG (six mice at week 10 and four mice at week 25). No anti-r-p185 antibodies were detectable in untreated 10- and 25-week-old BALB/c mice (data not shown).
Figure 7. Depletion of Treg cells unvels a CTL response to r-p185. Cytotoxicity displayed by 25-week-old BALB-neuT mice incubated with r-p185 IgG (D) or anti-CD25 IgG (E). A, mice were injected i.v. with 10^7 BALB/c Spc pulsed with r-p185 63-71 peptide and stained with 0.5 μg/ml CFSE or nonpulsed and stained with 0.5 μg/ml CFSE. Forty-eight hours later, Spc fluorescence was evaluated by flow cytometry and the percentage of lysis was calculated as described in Materials and Methods. B and C, 4-hour 51Cr release assay against r-p185^+ 3T3NKB cells (continuous lines) or r-p185^+ BALB 3T3 fibroblasts (dotted lines) with effector Spc restimulated in vitro with r-p185 63-71 peptide (B) or r-p185^+ 3T3NKB cells (C). A to C, five mice in each group; B and C, representative experiment.

References

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