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Primary Antitumor Immune Response Mediated by CD4+ T Cells

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Summary

Gene-targeted mice have recently revealed a role for lymphocytes and interferon-γ (IFNγ) in conferring protection against cancer, but the mechanisms remain unclear. Here, we have characterized a successful primary antitumor immune response initiated by naive CD4+ T cells. Major histocompatibility complex class II (MHC-II)-negative myeloma cells injected subcutaneously into syngeneic mice were surrounded within 3 days by macrophages that captured tumor antigens. Within 6 days, naive myeloma-specific CD4+ T cells became activated in draining lymph nodes and subsequently migrated to the incipient tumor site. Upon recognition of tumor-derived antigenic peptides presented on MHC-II by macrophages, the myeloma-specific CD4+ T cells were reactivated and started to secrete cytokines. T cell-derived IFNγ activated macrophages in close proximity to the tumor cells. Tumor cell growth was completely inhibited by such locally activated macrophages. These data indicate a mechanism for immunosurveillance of MHC-II-negative cancer cells by tumor-specific CD4+ T cells through collaboration with macrophages.

Introduction

The immune system has been proposed to specifically recognize and eliminate newly transformed cells (Burnet, 1970). A series of reports with gene-targeted mice have recently provided strong experimental support for this cancer immunosurveillance hypothesis. Mice deficient for IFNγ, IFNγ receptor, perforin, NKT cells, αβ T cells, γδ T cells, or both T and B cells (*Rag2*$^{-/-}$) are all more susceptible to spontaneous or carcinogen-induced cancer (Girardi et al., 2001; Kaplan et al., 1998; Shankaran et al., 2001; Smyth et al., 2000a, 2000b; Street et al., 2002). These studies in animals support earlier observations that humans with a reduced immune capacity are more prone to develop malignancies (Birkeland et al., 1995; Gatti and Good, 1971). However, the mechanisms of cancer immunosurveillance remain to be elucidated.

Our present knowledge of how T cells eliminate cancer is almost exclusively based on memory immune responses investigated with vaccinated mice (Gross, 1943; Lynch et al., 1972). Such studies have revealed the critical role of tumor-specific CD4+ T cells in helping cytotoxic CD8+ T cells to kill tumor cells (Ossendorp et al., 1998). In addition, CD4+ T cells themselves can reject tumors in the absence of CD8+ T cells (Fujiwara et al., 1984; Levitsky et al., 1994; Mumberg et al., 1999). It has been proposed that CD4+ T cells eliminate tumors through activation and recruitment of effector cells, including macrophages and eosinophils (Hung et al., 1998). Several studies suggest that cytokines such as IFNγ that are secreted by type I (Th1) CD4+ T cells might be involved in antitumor and antiangiogenic activities (Mumberg et al., 1999; Qin and Blankenstein, 2000). Unfortunately, despite these findings in vaccinated mice, it is presently unclear whether similar mechanisms apply in immunosurveillance, i.e., during the course of a primary antitumor immune response.

The tumor protective role of CD4+ T cells has been conceptually problematic since most tumor cells do not express MHC-II and thus cannot be directly recognized by tumor-specific CD4+ T cells. Therefore, rejection of MHC-II-negative tumor cells by CD4+ T cells is most likely dependent on professional antigen-presenting cells (APCs) that endocytose, process, and present tumor antigens on their MHC-II to tumor-specific CD4+ T cells. This hypothesis is supported by the observation that dendritic cells isolated from large tumors are loaded with tumor antigens and can activate tumor-specific CD4+ T cells (Dembic et al., 2000, 2001). However, it is presently not known whether APCs can efficiently activate tumor-specific CD4+ T cells during a primary immune response, when the tumor load is still very low.

In order to study the mechanisms of cancer immunosurveillance by CD4+ T cells, we used a T cell receptor (TCR)-transgenic mouse system (Lauritzsen et al., 1994). In these transgenic mice, T cells recognize a tumor-specific idiotopic (Id) peptide from the secreted immunoglobulin (Ig) L chain V region of the MOPC315 mouse myeloma, presented in the context of MHC-II I-E^{d} (Bogen et al., 1986). The TCR-transgenic mice were made homozygous for the severe combined immunodeficiency (SCID) mutation, which ensures the unique specificity of the T cells by preventing rearrangement of endogenous TCR chains (Bogen et al., 1995). The high frequency of naive tumor-specific CD4+ T cells in TCR-transgenic mice renders the mice resistant against subcutaneous (s.c.) injection with syngeneic MOPC315 tumor cells, whereas nontransgenic mice develop fatal tumors. Protection is Id specific, CD4+ T cell mediated, and does not require the presence of B cells, γδ T cells, and CD8+ T cells (Bogen et al., 1995; Lauritzsen et al., 1994). Importantly, MOPC315 lacks MHC-II and therefore cannot be directly recognized by transgenic Id-

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specific CD4+ T cells (Dembic et al., 2000; Lauritzsen and Bogen, 1993). However, in large tumors, infiltrating APCs are Id primed and stimulate Id-specific CD4+ T cells (Dembic et al., 2000, 2001). Rejection of MOPC315 by the Id-specific TCR-transgenic mice does not require immunization of the mice, and thus represents a genuine primary immune response.

It has been difficult to study the mechanisms of tumor rejection in Id-specific TCR-transgenic mice, because the myeloma cells could not be precisely localized in vivo after injection. To solve this, we have in this study embedded injected tumor cells in a collagen gel (Matrigel), which enabled us to analyze the early interactions between tumor cells and infiltrating cells from the host, during a primary antitumor immune response.

Results

TCR-Transgenic SCID Mice Are Protected against MOPC315 Myeloma Cells Injected in Phosphate-Buffered Saline or Matrigel

In accordance with a previous report (Bogen et al., 1995), Id-specific TCR-transgenic SCID mice were resistant against s.c. challenge with MOPC315 cells injected in phosphate-buffered saline (PBS), whereas nontransgenic SCID littermates developed fatal tumors (Figure 1A). To estimate the kinetics of rejection, serum concentrations of myeloma protein M315 were measured at several time points after injection by enzyme-linked immunosorbent assay (ELISA) (Figure 1B). In most control SCID mice, M315 levels increased exponentially, reflecting progressive s.c. tumor growth (Figure 1B, left). By contrast, in all TCR-transgenic SCID mice, M315 levels were low (<350 ng/ml) throughout the experiment (Figure 1B, right). In 9 out of these 18 TCR-transgenic SCID mice, M315 remained undetectable in serum (detection level: 1 ng/ml). In the other 9 mice, the serum concentration of M315 increased slowly until days 8–11, suggesting that the tumor cells underwent a limited in vivo expansion during the first days after injection. After day 11, however, serum M315 levels decreased in all TCR-transgenic SCID mice, reflecting the elimination of the tumor cells. These data strongly suggest that the effector functions of the antitumor immune response are activated well before day 11 after injection, and that the tumor cells are completely rejected by day 15.

In order to precisely localize the injected tumor cells in the host, we embedded MOPC315 cells in a collagen gel (Matrigel) that is soluble at +4°C but gels at body temperature, resulting in a plug that can easily be identified in vivo (Kleinman et al., 1986). Another advantage of this technique is that it traps the infiltrating host cells (see below). TCR-transgenic SCID mice were effectively protected against MOPC315 injected in Matrigel for more than 50 days after injection (Figure 1C). However, most transgenic mice failed to completely reject the myeloma cells injected in Matrigel, as revealed by low but sustained levels of serum M315 (Figure 1D). As a consequence of this incomplete rejection, slow-growing tumors developed in three out of seven TCR-transgenic SCID mice as late as 60–90 days after the injection (Figure 1C). As with MOPC315 injected in PBS, the antitumor immune response against MOPC315 in Matrigel was rapid, since as early as 12 days after injection, serum M315 levels were significantly lower in transgenic mice compared to SCID controls (Figure 1D, right).

Tumor-Specific CD4+ T Cells Become Activated in Draining Lymph Nodes, Migrate to the Incipient Tumor Site, and Secrete Cytokines

Proliferation of tumor-specific CD4+ T cells was first observed in the lymph node (LN) draining the injection site at day 3 and dramatically increased at day 6 after injection of MOPC315 in either Matrigel or PBS (Figure 2A and data not shown). This clonal expansion was associated with upregulation of the activation marker CD69 on most tumor-specific CD4+ T cells (Figure 2A). The immune response was local, as similar changes were not observed in nondraining LN or spleen (data not shown; also, see below). The T cell activation was not due to Matrigel per se because injection of cell-free Matrigel gave no response on day 6. Moreover, T cell activation was Id specific since no response was seen after injection with the control J558 myeloma, which secretes a monoclonal IgA with V regions different from those of MOPC315 (Figure 2A). This is in accordance with previous reports showing that the TCR-transgenic mice reject MOPC315, but not J558 cells (Bogen et al., 1995; Lauritzsen et al., 1994).

From days 3–9 after tumor cell injection, the tumor-specific CD4+ T cells differentiated in draining LN from naive to memory phenotype: the cells increased in size (blast formation), upregulated surface CD11a and CD44 molecules, downregulated CD62L, and synthesized DNA (i.e., incorporated bromodeoxyuridine, BrdU) (Figure 2C, left and data not shown). Since $CD44^{hi}CD62L^{lo}$ T cells have the capacity to enter nonlymphoid tissues, these data prompted us to analyze the cellular content of MOPC315-containing Matrigel plugs. At day +6, a small but distinct population of Matrigel-infiltrating CD69+ tumor-specific T cells could be detected (Figure 2B). At day +9, Matrigel-infiltrating tumor-specific T cells were more frequent and had a typical memory phenotype (enlarged size, $CD11a^{hi}$, $CD44^{hi}$, $CD62L^{lo/-}$, $BrdU^{+}$) (Figure 2C, right). Importantly, these Matrigel-infiltrating tumor-specific T cells were producing the Th1 cytokines IFNγ and tumor necrosis factor α (TNFα) at day +11 (Figure 2D). In the same experiment, interleukin-2, but not granulocyte/macrophage colony-stimulating factor (GM-CSF), was detected in Matrigel-infiltrating tumor-specific CD4+ T cells (data not shown). These results demonstrate that 11 days are sufficient for the priming of tumor-specific CD4+ T cells in draining LN, the migration of primed T cells into the tissue in which the tumor cells are located, and the secretion of cytokines.

Massive Recruitment of Host Cells toward the Injected Myeloma Cells

The early in vivo interactions between the tumor cells and cells of the immune system were visualized by immunostaining of MOPC315-containing Matrigel plugs in TCR-transgenic SCID mice (Figure 3). Figure 3A

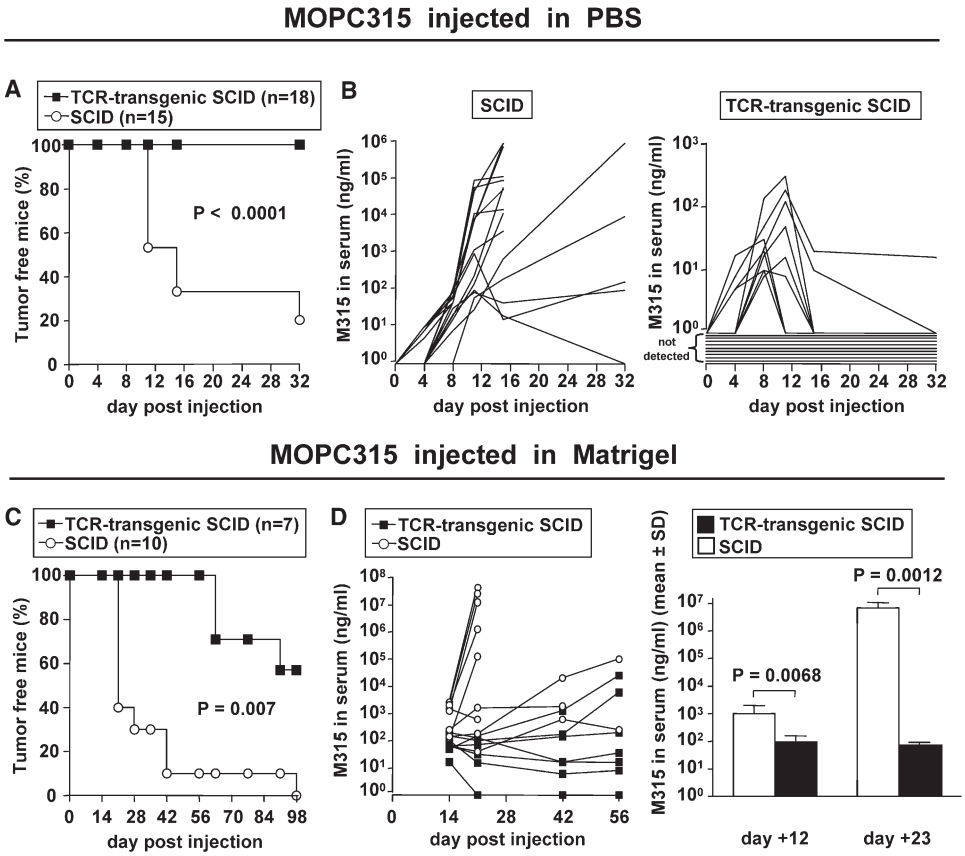

Figure 1. TCR-Transgenic SCID Mice Are Protected against MOPC315 Myeloma Cells Injected in PBS or Matrigel

(A–D) TCR-transgenic SCID and SCID littermates were injected s.c. with MOPC315 cells in (A and B) PBS or in (C and D) Matrigel. (A and C) Tumor development was followed by palpation, and (B and D) serum myeloma protein M315 concentration was measured by ELISA. Mice with large tumors (diameter >10 mm) were euthanized. P values were calculated with the (A and C) logrank test and the (D) Mann-Whitney test.

shows a s.c. day +6 Matrigel plug containing islets of tumor cells (arrows). Recruitment of blood leukocytes toward the MOPC315-containing Matrigel plug was suggested by the appearance of nucleated cells within blood vessels at the periphery of the plug (day +3, Figures 3B and 3C). At day +6, the nucleated (Hoechst$^+$) cells inside the Matrigel plug can basically be divided into two distinct populations: (i) the MOPC315 cells growing in islets and stained by the myeloma marker CD138 (syndecan-1), and (ii) the host cells that essentially all expressed MHC-II (Figure 3D). These MHC-II$^+$ host cells formed a dense layer covering the edge of the myeloma-containing Matrigel plug 3–6 days after injection (arrows in Figures 3D and 3F). Numerous MHC-II$^+$ host cells were seen penetrating the Matrigel plug, of which several were in close contact with the CD138$^+$ myeloma cells (arrows in Figure 3E). The Matrigel-infiltrating MHC-II$^+$ cells coexpressed the macrophage marker F4/80 (day +3, Figure 3F). Figure 3G shows a MHC-II$^+$ cell crossing the endothelium of a blood vessel inside a Matrigel plug. Collectively, these data suggest that there is a massive recruitment of host MHC-II$^+$ macrophages toward the tumor cells. These macrophages are most likely derived from blood monocytes that extravasated mainly from vessels situated at the periphery of the plug, but also from vessels surrounded by the gel. Additionally, infiltrating T cells could be detected in Matrigel sections, but they were much fewer than the macrophages. Importantly, some tumor-specific T cells apparently made contact with MHC-II$^+$ macrophages in the MOPC315-containing Matrigel plug (Figure 3H).

Matrigel-Infiltrating MHC-II$^+$ Cells Are Mostly Macrophages

We next used flow cytometry to characterize the Matrigel-infiltrating MHC-II$^+$ cells 1–6 days after injection. For these experiments, green fluorescent protein (GFP)-transduced MOPC315 cells were used, allowing a simple and effective detection of tumor cells. Analysis of the cellular content of a Matrigel plug at day +5 revealed a massive infiltration of cells expressing the CD11b (Mac-1) myeloid cell marker (Figure 4A). MHC-II expression was almost exclusively restricted to these CD11b$^+$ cells (R1 in Figure 4A), whereas GFP$^+$ MOPC315 cells were MHC-II negative (R3). A large population of CD11b$^-$GFP$^-$ cells that were essentially devoid of MHC-II expression was also detected in Matrigel (Figure 4A, R2). Most of these triple-negative cells were most likely MOPC315 cells that had lost GFP expression (un-

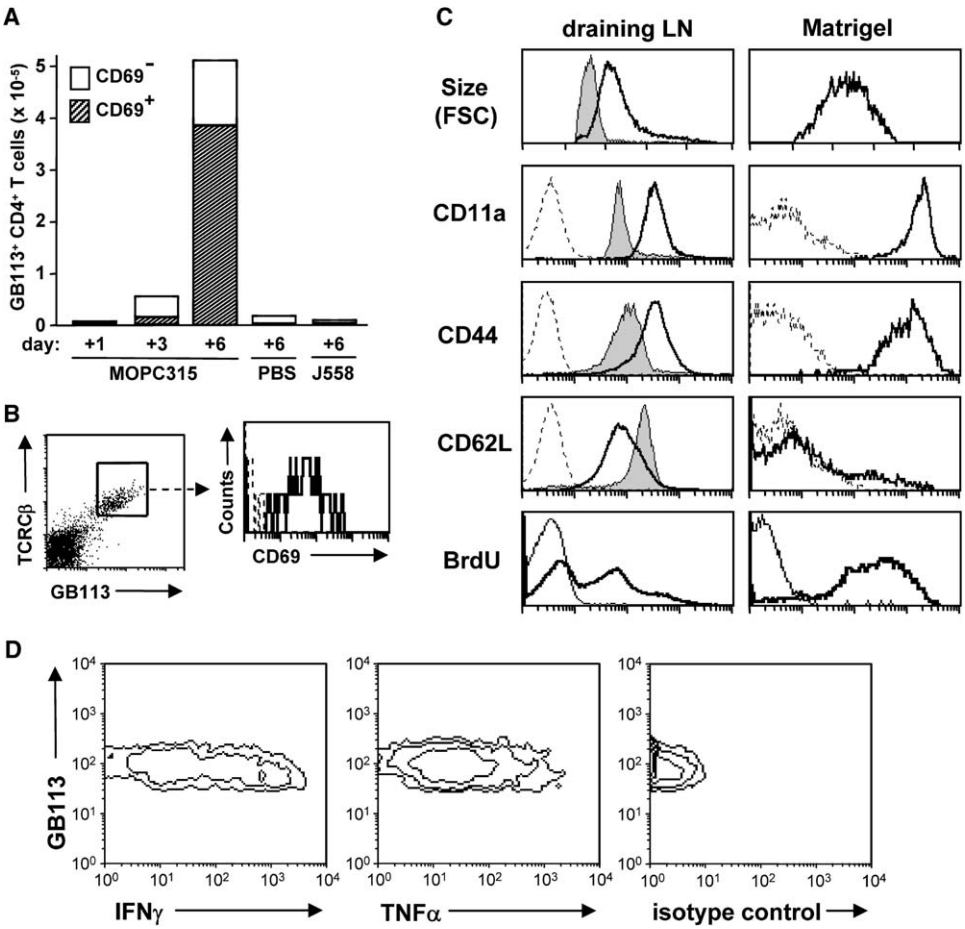

Figure 2. Naive Tumor-Specific CD4+ T Cells Become Activated in Draining LN, Migrate to the Incipient Tumor Site, and Produce Cytokines

TCR-transgenic SCID mice were injected s.c. with Matrigel containing either tumor cells (MOPC315 or control J558) or PBS only and were analyzed by flow cytometry.

(A) Numbers of tumor-specific (detected by the clonotype-specific GB113 mAb) CD4+ T cells in draining LN 1–6 days after injection. Each column represents the mean of three mice (pooled organs). Hatched areas represent the proportion of activated (CD69+) cells.

(B) At day +6, a MOPC315-containing Matrigel plug was digested with collagenase/DNase to release the cells, and infiltrating tumor-specific T cells were detected by double staining with anti-TCRCβ and GB113 mAb. Insert, CD69 expression on gated TCRCβ+GB113+ T cells.

(C) Phenotype of tumor-specific CD4+ T cells in draining LN and within the MOPC315-containing Matrigel plug at day +9 (boldface line). For comparison, LN cells from naive, uninjected TCR-transgenic SCID mice were stained and gated similarly (shaded area). The specificity of the BrdU staining was controlled by using mice not treated with BrdU (thin line).

(D) Matrigel-infiltrating tumor-specific CD4+ T cells were stained for intracellular IFNγ and TNFα at day +11, after a 6 hr in vitro stimulation with ionomycin/PMA in the presence of monensin. The dotted line in (B) and (C) indicates an isotype-matched control mAb.

published data), compatible with the immunostaining data which show that essentially all MHC-II-negative cells in Matrigel stain positively for the myeloma marker CD138 (Figure 3D). Further characterization of the Matrigel-infiltrating CD11b+ cells identified the cells as typical macrophages: they expressed CD11a (LFA-1 α chain), CD54 (ICAM-1), CD80, CD86 and Mac-3 (Figure 4B), while they were negative for CD45R/B220, CD4 and CD8 (not shown). Interestingly, a minority (4%–12%) of the Matrigel-infiltrating CD11b+ cells expressed CD11c+, indicating a small subset of dendritic cells (Figure 4B).

Tumor-Specific CD4+ T Cells Activate Matrigel-Infiltrating Macrophages

The immunostaining data revealed that some tumor-specific CD4+ T cells made close contact with macrophages inside MOPC315-containing Matrigel plugs (Figure 3H). A functional consequence of such an interaction could be the activation of the macrophages by the CD4+ T cells. Indeed, after MOPC315 injections, levels of the activation marker MHC-II on Matrigel-infiltrating macrophages were dramatically increased in TCR-transgenic SCID mice as compared to nontransgenic SCID mice (Figure 4C). Moreover, two additional macrophage activation markers (CD11a and CD54) were upregulated on infiltrating macrophages in TCR-transgenic SCID mice (Figure 4B). This prompted us to investigate the kinetics of macrophage recruitment and activation. Large numbers of macrophages were found in Matrigel plugs containing MOPC315 as early as 3 days after injection, but, at this time point, MHC-II expression was not upregulated (Figure 4D). In contrast, at day +6, most Matrigel-infiltrating CD11b+ cells had upregulated MHC-II in TCR-transgenic mice (Figure 4D), correlating with the influx of tumor-specific CD4+

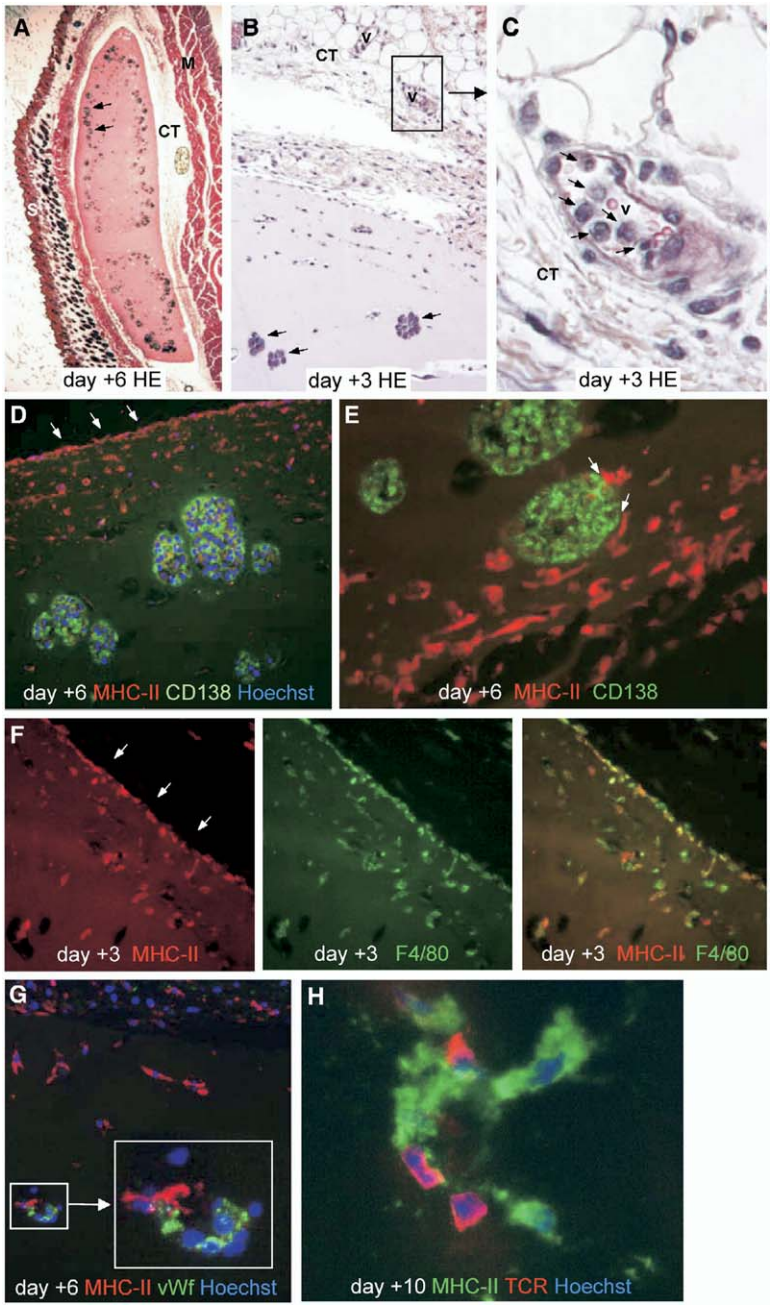

Figure 3. Recruitment of Host Cells toward Myeloma-Containing Matrigel Plugs

(A–H) TCR-transgenic SCID mice were injected s.c. in the flank with MOPC315 cells in Matrigel. Tissue samples were taken for (A–C) H+E staining or (D–H) immunohistochemistry 3–10 days after injection. (A) Subcutaneous day +6 Matrigel plug containing MOPC315 cells. Examples of tumor islets are shown by arrows. (B) Blood vessels in the vicinity of a day +3 Matrigel plug (arrows, islets of myeloma cells). (C) Higher magnification of the insert in (B) showing the high number of nucleated cells within the vessel (arrows). (D and E) MHC-II+ (red) host cells infiltrate a day +6 Matrigel plug containing CD138+ (green) MOPC315 cells. Arrows in (D) show the border of the plug. Arrows in (E) show contacts between MHC-II+ cells and CD138+ tumor cells. (F) The Matrigel-infiltrating MHC-II+ (red) cells express the macrophage marker F4/80 (green) at day +3. Arrows show the border of the plug. (G) MHC-II+ (red) cells penetrate deep into a day +6 Matrigel plug. The edge of the plug is at the top of the image. The insert shows a MHC-II+ cell crossing the endothelium of a von Willebrand factor+ (vWf+, green) blood vessel in the interior of a Matrigel plug. (H) In a day +10 MOPC315-containing Matrigel plug, tumor-specific T cells (TCRVβ8+, red) can be seen contacting MHC-II+ (green) cells. CT, soft connective tissue; M, striated muscle; S, skin; v, blood vessel. Hoechst (blue) stains cell nuclei.

T cells at the same time point (Figure 2B). Interestingly, the recruitment of macrophages was dependent on the presence of myeloma cells and was not caused by the Matrigel itself since very few macrophages infiltrated cell-free Matrigel plugs (Figure 4D).

In order to demonstrate that the observed macrophage activation was mediated by the tumor-specific CD4+ T cells, we performed two in vivo experiments with blocking monoclonal antibodies (mAb). First, we used an anti-CD4 mAb to deplete CD4+ T cells in TCR-transgenic SCID mice. The activation of Matrigel-infiltrating macrophages was completely blocked in such CD4+ T cell-depleted mice (Figure 5A). In a second experiment, we took advantage of an anti-MHC-II I-E mAb (Dembic et al., 2004) to block the activation of tumor-specific CD4+ T cells in draining LN (Figure 5B). Such blocking of T cell activation inhibited the migration of T cells into Matrigel (Figure 5C), indicating that priming in draining LN is a prerequisite for the migration of tumor-specific CD4+ T cells to the incipient tumor site. Moreover, blocking of T cell activation and migration with anti-MHC-II mAb completely inhibited macrophage activation in the Matrigel plugs, as measured by MHC-II I-A and CD11a levels (Figures 5D and 5E).

Antigen-Specific Activation of Matrigel-Infiltrating Macrophages by CD4+ T Cells

The antigen specificity of macrophage activation by CD4+ T cells was tested by injecting Id-specific TCR-transgenic SCID mice with either MOPC315 or the con-

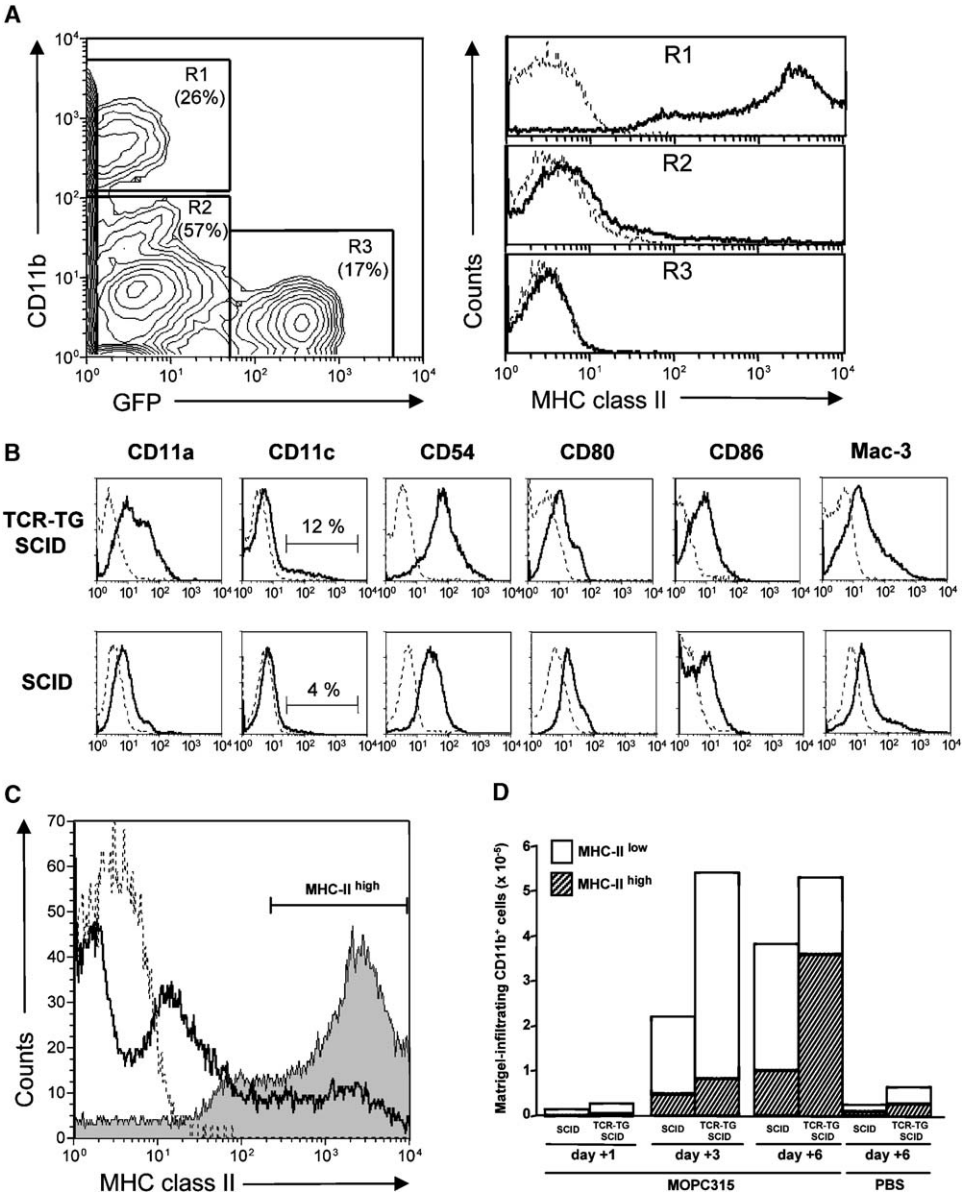

Figure 4. Matrigel-Infiltrating Macrophages Become Activated in TCR-Transgenic Mice

(A) A TCR-transgenic SCID mouse was injected with GFP-labeled MOPC315 cells in Matrigel. The cellular content of the Matrigel plug was analyzed by flow cytometry at day +5, revealing three distinct populations: CD11b$^+$ cells (R1), GFP$^-$CD11b$^-$ cells (R2), and GFP$^+$ tumors cells (R3). MHC-II expression was almost exclusively restricted to the CD11b$^+$ population (R1), while the myeloma cells (R3) were negative for MHC-II.

(B) TCR-transgenic SCID or SCID mice were injected with Matrigel containing MOPC315. The expression of various markers on gated Matrigel-infiltrating CD11b$^+$ cells was analyzed by flow cytometry at day +6.

(C) MOPC315 cells were injected s.c. in Matrigel, and MHC-II expression on Matrigel-infiltrating CD11b$^+$ cells was measured by flow cytometry at day +5 in a TCR-transgenic SCID mouse (shaded area) as compared to a SCID mouse (boldface line).

(D) TCR-transgenic SCID or SCID mice were injected with either MOPC315-containing Matrigel or cell-free, PBS-containing Matrigel. At various time points after injection, the Matrigel-infiltrating CD11b$^+$ cells were counted and divided into MHC-II high (hatched area) or low (blank area) expressers, as defined in (C). Each column represents the mean of three mice (pooled organs). T cell activation in draining LN was analyzed in parallel and the data are represented in Figure 2A. Dotted lines in (A), (B), and (C) indicate an isotype-matched control mAb. TG, transgenic.

trol J558 myeloma (Figure 6A). In contrast to MOPC315, injections with J558 did not result in macrophage activation in TCR-transgenic SCID mice (Figure 6A). In the same experiment, nontransgenic SCID mice were injected with MOPC315 and J558, and this confirmed that CD4$^+$ T cells are needed for macrophage activation in MOPC315-containing Matrigel (Figure 6A). These results suggest that macrophage activation is the result of CD4$^+$ T cell recognition of tumor antigens presented on MHC-II molecules by Matrigel-infiltrating macro-

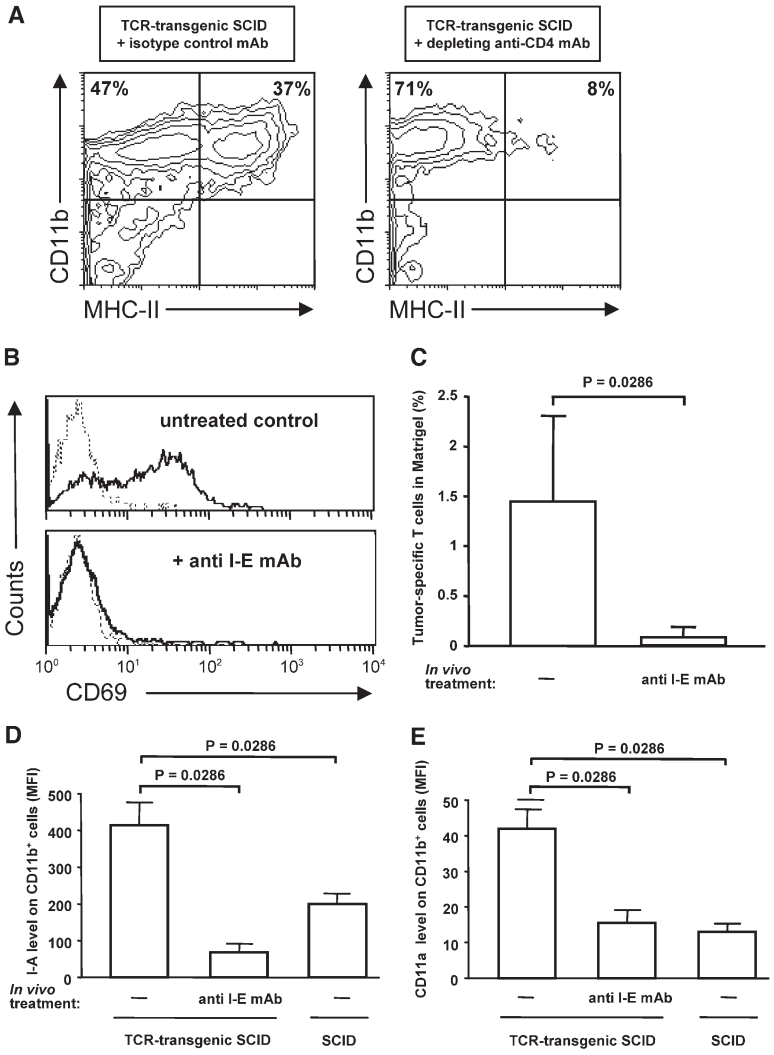

Figure 5. Primed Tumor-Specific CD4+ T Cells Migrate to the Incipient Tumor Site, Where They Activate Macrophages

(A) TCR-transgenic SCID mice were injected (200 μg i.p.) twice a week with either depleting anti-CD4 mAb (GK1.5) or with isotype-matched control mAb (KT4-10), starting 1 week before the injection of MOPC315 in Matrigel. MHC-II expression on Matrigel-infiltrating CD11b+ macrophages was analyzed at day +6. Data for one representative mouse in each group are shown.

(B–E) TCR-transgenic SCID (n = 8) or non-transgenic SCID mice (n = 4) were injected with MOPC315 cells in Matrigel. Starting at day +2, four TCR-transgenic SCID mice were treated daily with an anti-MHC-II I-E blocking mAb (14-4-4S, 200 μg i.p. + 200 μg s.c.). The remaining mice (four TCR-transgenic SCID and four SCID) were left untreated. At day +7, draining LN and Matrigel plugs were analyzed by flow cytometry. (B) CD69 expression on gated tumor-specific (GB113+) CD4+ T cells in draining LN of a nontreated (top) or anti-I-E-treated (bottom) TCR-transgenic SCID mouse. Similar data were obtained for each of the four mice per group. (C) Percentage of tumor-specific (GB113+) T cells in Matrigel plugs in untreated or anti-I-E-treated TCR-transgenic SCID mice (mean ± SD). (D and E) Expression of the activation markers MHC-II I-A (D) and CD11a (E) on Matrigel-infiltrating CD11b+ cells in the three experimental groups (mean ± SD). It should be noted that the 14-4-4S mAb used to treat the mice is specific for MHC-II I-E and is therefore not expected to interfere directly with flow cytometric measurements of the levels of MHC-II I-A with the NIMR-4 mAb. P values were calculated with the Mann-Whitney test.

phages. We therefore examined whether Matrigel-infiltrating macrophages could present tumor-derived Id peptides. Macrophages that had infiltrated MOPC315-containing Matrigel plugs were purified and tested for their ability to spontaneously stimulate Id-specific CD4+ T cells in vitro. A modest proliferation of tumor-specific CD4+ T cells could be detected in the absence of added peptide, indicating an in vivo loading of the macrophages (Figure 6B). Addition of exogenous synthetic Id peptide to the cultures further improved the proliferation, demonstrating that the Matrigel-infiltrating macrophages were potent APCs (Figure 6B). Although Matrigel-infiltrating macrophages upregulate MHC-II in TCR-transgenic mice (Figure 6A), no difference was observed in antigen presentation capacity between macrophages isolated from TCR-transgenic SCID versus SCID mice at day +5 (Figure 6B).

The antigen specificity of macrophage activation by CD4+ T cells can in theory take place either (i) in draining LN, during the initial T cell priming, or (ii) in the incipient tumor tissue, when CD4+ T cells interact with macrophages. We have demonstrated above that CD4+ T cell priming in LN was indeed antigen specific (Figure 2A). To assess the role of antigen presentation in the malignant tissue, we designed an experiment in which tumor-specific CD4+ T cells would be able to meet their cognate antigen in the LN (and be primed), but not in the malignant tissue. For this purpose, TCR-transgenic SCID mice were injected with MOPC315-containing Matrigel on the right flank and J558-containing Matrigel on the left flank (Figures 6C–6E). At day 6 after injection, Matrigel plugs and draining LN were analyzed individually. Several observations could be made from this experiment. First, Id-specific CD4+ T cells became activated in the right flank LN, draining MOPC315-containing Matrigel, but not in the left flank LN, draining J558-containing Matrigel (Figure 6C, upper part). This demonstrates that the initial priming of tumor-specific CD4+ T cells is taking place locally, in the LN draining the malignant tissue, rather than systemically. Second, primed Id-specific CD4+ T cells migrated to the same extent into both MOPC315- and J558-containing Matrigel plugs (Figure 6D, left). This reveals that, in contrast to the priming in LN, the migration of primed CD4+ T cells into tumor tissues is not antigen specific. Third, macrophages were recruited to the same extent into

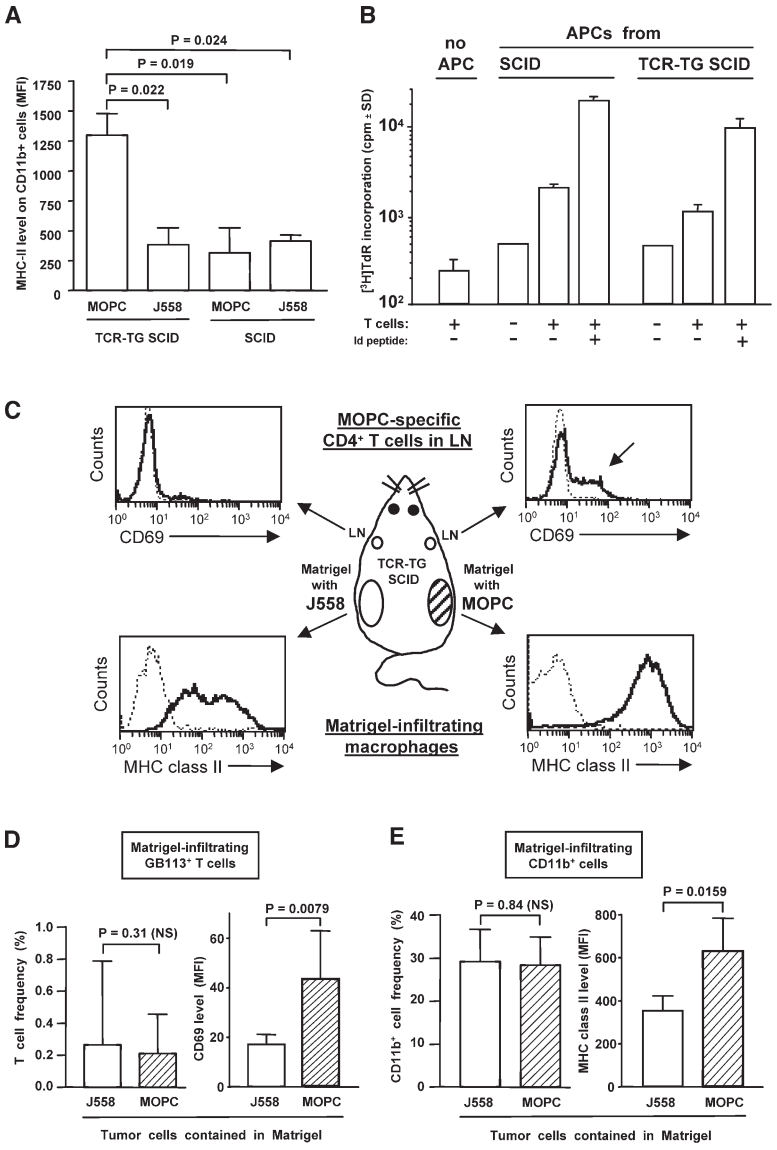

Figure 6. Antigen-Specific Activation of Tumor-Infiltrating Macrophages by CD4+ T Cells

(A) TCR-transgenic SCID or nontransgenic SCID mice (four mice per group) were injected with Matrigel containing either MOPC315 or J558 cells. MHC-II expression (mean ± SD) on gated Matrigel-infiltrating CD11b+ cells at day +6 is represented.

(B) TCR-transgenic SCID or SCID mice were injected with Matrigel containing MOPC315. Matrigel-infiltrating CD11b+ cells were purified at day +5 and tested for their ability to stimulate in vitro the proliferation of Id-specific CD4+ T cells, with or without the addition of Id peptide into the cultures.

(C–E) TCR-transgenic SCID mice (n = 5) were injected s.c. with MOPC315-containing Matrigel on the right flank and J558-containing Matrigel on the left flank. At day +6, Matrigel plugs and draining LN were analyzed individually. (C) A representative mouse. Upper part, expression of CD69 on gated CD4+GB113+ T cells in LN draining the left and the right flank. Lower part, expression of MHC-II on gated CD11b+ cells that had infiltrated the Matrigel plugs containing either J558 (left) or MOPC315 (right). The dotted line indicates an isotype-matched control mAb. (D) Frequency and CD69 expression levels of Matrigel-infiltrating GB113+TCRβ+ T cells (mean ± SD). (E) Frequency and MHC-II expression levels of Matrigel-infiltrating CD11b+ cells (mean ± SD). P values were calculated with the (A) unpaired t test and the (D and E) Mann-Whitney test. MFI, mean fluorescence intensity; NS, not statistically significant; TG, transgenic.

MOPC315- and J558-containing Matrigel plugs (Figure 6E, left). Fourth, macrophages were activated in MOPC315-containing Matrigel, but not in Matrigel with J558, demonstrating the importance of antigen presentation in situ for T cell-mediated macrophage activation (Figures 6C, lower part, and 6E, right). Fifth, the levels of CD69 on Matrigel-infiltrating T cells were higher in the MOPC315-containing Matrigel plug as compared to the J558-containing Matrigel plug. This strongly suggests that T cell recognition of tumor-derived peptides presented on MHC-II by macrophages results in reactivation of the tumor-specific CD4+ T cells in situ at the incipient tumor site (Figure 6D, right).

IFNγ Is Critical for T Cell-Mediated Macrophage Activation and Tumor Rejection

We have shown in Figure 2D that Matrigel-infiltrating tumor-specific CD4+ T cells produce IFNγ, which is a potent macrophage activation factor (Steeg et al., 1982). To test whether the observed activation of Matrigel-infiltrating macrophages was dependent on IFNγ, TCR-transgenic SCID mice were injected with a blocking anti-IFNγ mAb. Such a treatment affected neither T cell activation in draining LN nor T cell migration into the Matrigel plug (Figure S1). In contrast, T cell-mediated activation of Matrigel-infiltrating macrophages was completely inhibited by blocking IFNγ (Figure 7A). Notably, macrophage activation could be restored in nontransgenic SCID mice by s.c. injection with mouse recombinant IFNγ (Figure 7A). Thus, IFNγ is critical for activation of the macrophages that infiltrate the MOPC315-containing Matrigel plugs.

Macrophage activation by IFNγ is referred to as the classical activation pathway and is characterized by upregulation of surface FcγRII/III. A number of alternative pathways for macrophage activation, which are associated with upregulation of receptors like FcεRII

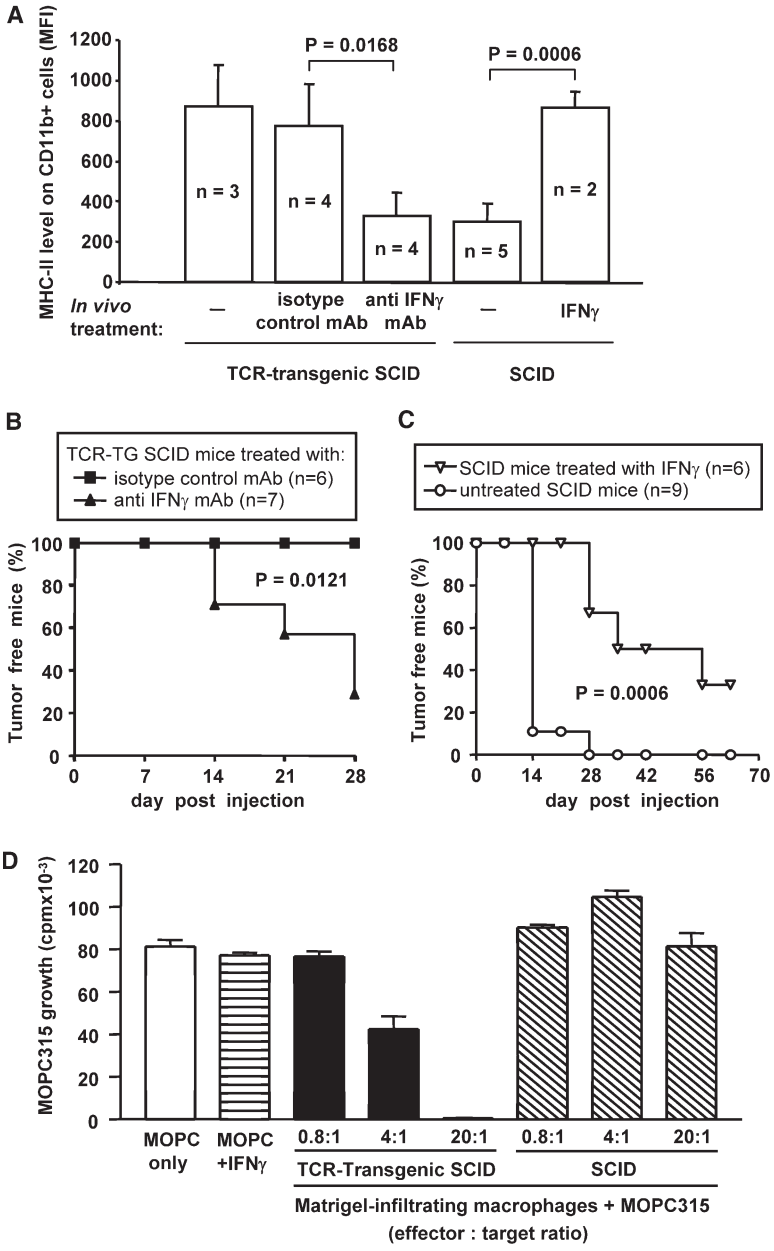

Figure 7. IFNγ Is Critical for Macrophage Activation and Tumor Rejection

(A) TCR-transgenic SCID and SCID mice were injected with Matrigel containing MOPC315 cells. At day +1 and +4, groups of mice were treated with either blocking anti-IFNγ mAb (XMG1.2, 100 μg i.p. + 100 μg s.c.), isotype-matched control mAb (187.1, 100 μg i.p. + 100 μg s.c.), or mouse recombinant IFNγ (2×10^3 U, s.c.), or they were left untreated. At day +6, MHC-II expression on Matrigel-infiltrating CD11b+ cells was analyzed by flow cytometry (mean ± SD).

(B) TCR-transgenic SCID mice were challenged s.c. with MOPC315 cells in PBS and injected three times a week with either blocking anti-IFNγ mAb or isotype-matched control mAb (100 μg i.p. + 100 μg s.c.). Tumor growth was recorded over time.

(C) SCID mice were challenged s.c. with MOPC315 cells in PBS and injected s.c. three times a week with recombinant mouse IFNγ (2×10^3 U, s.c.) or were left untreated. Palpable tumors were recorded over time.

(D) TCR-transgenic SCID and SCID mice were injected with Matrigel containing MOPC315. Matrigel-infiltrating CD11b+ cells were purified at day +7 and tested at various effector to target ratios for their ability to suppress the proliferation (mean cpm ± SD) of MOPC315 cells in vitro in a growth inhibition assay. In the same experiment, the direct cytotoxicity of IFNγ (5000 U/ml) on MOPC315 was tested. P values were calculated with the (A) unpaired t test and the (B and C) logrank test.

(CD23), scavenger RI (CD204), and mannose R (CD206), have been described (Mantovani et al., 2004). Flow cytometry analysis confirmed that Matrigel-infiltrating macrophages in TCR-transgenic SCID mice have a classical activation phenotype (FcγRII/III^{hi}, CD23^{-}, CD204$^{lo/-}$, CD206 $^{lo/-}$), in accordance with their IFNγ-mediated activation (Figure S2).

The observations reported above prompted us to investigate the importance of IFNγ in tumor rejection. In a first experiment, blockage of IFNγ rendered the TCR-transgenic SCID mice susceptible to MOPC315 tumor development, revealing that the antitumor response was dependent on IFNγ (Figure 7B). In a second complementary experiment, s.c. injection with mouse recombinant IFNγ was shown to significantly delay MOPC315 tumor growth in SCID mice (Figure 7C). This indicates that the antitumor function of tumor-specific CD4+ T cells can at least partly be substituted with local injection of IFNγ.

T Cell-Activated Macrophages Suppress Tumor Cell Growth

Our data revealed that the primary antimyeloma immune response is associated with secretion of IFNγ by tumor-specific CD4+ T cells, resulting in activation of macrophages in close proximity to the tumor cells. This prompted us to test whether IFNγ and T cell-activated macrophages could directly exert tumor suppressive functions. Addition of IFNγ in high concentration to MOPC315 cells in vitro had no effect on cell proliferation, demonstrating that IFNγ per se was not directly cytotoxic to the myeloma cells (Figure 7D). Similarly,

Matrigel-infiltrating macrophages isolated from control SCID mice had no influence on MOPC315 in vitro growth (Figure 7D). In striking contrast, Matrigel-infiltrating macrophages isolated from TCR-transgenic SCID mice at day +7 were able to completely inhibit the in vitro proliferation of MOPC315 cells in a dose-dependent manner (Figure 7D). These results demonstrate that Matrigel-infiltrating macrophages that have been activated in vivo by tumor-specific $CD4^+$ T cells can effectively inhibit the growth of MOPC315 cells in vitro. Since the suppression of tumor cell growth was observed in a short-term (2.5 days) ex vivo assay, it is likely that the activated macrophages exert the same inhibitory function in vivo.

Discussion

A series of recent reports have shed light on the various strategies used by the immune system to recognize and eliminate newly transformed cells. A number of lymphocyte subsets (like $CD8^+$ T cells, $\gamma\delta$ T cells, NK cells, and NKT cells) and effector mechanisms (such as IFNγ, perforin, and TRAIL) have been shown to be critical for immunosurveillance against various types of malignancies (Girardi et al., 2001; Kaplan et al., 1998; Shankaran et al., 2001; Smyth et al., 2000a, 2000b; Street et al., 2002; Takeda et al., 2002).

To our knowledge, our data provide the first description of a successful primary antitumor immune response mediated by $CD4^+$ T cells. Several obstacles have previously hindered such a study: (i) the exact time and localization of cellular transformation leading to malignancy are close to impossible to determine, (ii) T cells specific for a given antigen are extremely infrequent ($<10^{-6}$) and therefore difficult to detect, before they become activated and clonally expand, and (iii) when immunosurveillance is successful, transformed cells are rapidly eliminated by the immune system, leaving no tumor specimen for investigation. We circumvented these problems by transplanting syngeneic tumor cells embedded in a collagen gel into TCR-transgenic mice with high numbers of naive tumor-specific $CD4^+$ T cells.

Our data illustrate that naive tumor-specific $CD4^+$ T cells can efficiently be primed in the draining LN within 3–6 days after s.c. injection of tumor cells. This initial activation results in changes in the displayed cell surface molecules, allowing the tumor-specific $CD4^+$ T cells to leave the lymphoid organ and to migrate to the incipient tumor site.

Characterization of the early immunological events at the incipient tumor site was made possible by embedding the injected myeloma cells in Matrigel. Matrigel is a soluble basement membrane that is derived from a murine tumor and therefore represents a genuine tumor cell microenvironment (Kleinman et al., 1986). Matrigel constituents (mainly laminin, type IV collagen, and heparan sulfate proteoglycan) are physiological components of the extracellular matrix and thus are not expected to hinder migration of leukocytes. Indeed, already 3 days after injection, myeloma-containing Matrigel plugs were massively infiltrated by macrophages, apparently recruited by chemoattractants secreted by tumor cells (Bottazzi et al., 1983). Tumor-specific $CD4^+$ T cells, previously primed in the LN, arrived a few days later and interacted with the macrophages inside the Matrigel plug.

MOPC315, like most cancer cells, lacks MHC-II and therefore cannot be directly recognized by tumor-specific $CD4^+$ T cells. However, our data reveal that the antigen specificity of the anti-MOPC315 immune response is ensured at two checkpoints: (i) during the priming of naive tumor-specific $CD4^+$ T cells in draining LN, and (ii) in the malignant tissue, when tumor-specific $CD4^+$ T cells are reactivated by infiltrating macrophages that present tumor-derived peptides on their MHC-II molecules. Macrophages were identified as the predominant APCs at the incipient tumor site. This finding contrasts with previous studies in large tumors in which dendritic cells were reported to activate tumor-specific T cells (Chiodoni et al., 1999; Dembic et al., 2000, 2001). Our results suggest that the microenvironment surrounding newly transformed cells being macrophage enriched might be quite different from that of established tumors, which is dendritic cell enriched.

Upon recognition of tumor-derived antigenic peptides presented on MHC-II by macrophages, the myeloma-specific $CD4^+$ T cells were reactivated and started to secrete IFNγ and TNFα in close proximity to the tumor cells. T cell-derived IFNγ was shown to be required for MOPC315 rejection, providing support for the important role of IFNγ in cancer immunosurveillance (Kaplan et al., 1998; Street et al., 2002). The function of TNFα was not investigated in the present study. An interesting possibility is that TNFα may act in synergy with IFNγ for the activation of tumoricidal macrophages, as previously shown in vitro (Hori et al., 1987). However, it should be noted that the role of TNFα in cancer is a subject of debate, since both tumor-promoting and tumor-suppressing effects of TNFα have been reported (Balkwill, 2002). Conceivably, FasLigand expression on activated tumor-specific $CD4^+$ T cells could also contribute to tumor rejection. However, TCR-transgenic mice homozygous for the *gld* mutation are still able to reject MOPC315 cells, demonstrating that FasLigand is dispensable for tumor protection (unpublished data).

Our results suggest that an important role of T cell-derived IFNγ is to activate the antitumor effector functions of macrophages. Activated macrophages have been reported to secrete a number of compounds with tumoricidal activity, like nitric oxide and reactive oxygen intermediates (Mantovani et al., 2004). The molecular mechanisms whereby activated macrophages inhibit MOPC315 cell growth in our in vivo model are currently under investigation. IFNγ was shown to be crucial for macrophage activation, but additional antitumor effects of IFNγ were not excluded. IFNγ did not directly kill or inhibit the proliferation of MOPC315 cells in vitro. However, IFNγ could inhibit tumor growth in vivo by inducing the production of antiangiogenic chemokines by the tumor cells or by stromal cells within the tumor microenvironment, as reported in other systems (Coughlin et al., 1998; Qin and Blankenstein, 2000).

It is well established that solid tumors are infiltrated by macrophages, but the function of these tumor-associated

macrophages (TAMs) is controversial. Initial studies revealed that peritoneal macrophages from immunized mice could kill tumor cells in vitro, and the reaction was shown to be immunologically specific (Evans and Alexander, 1970). Subsequent in vitro studies demonstrated that macrophages could be rendered tumoricidal by treatment with IFNγ (Schreiber et al., 1983). However, extensive infiltration of tumors by macrophages is often associated with poor prognosis (Bingle et al., 2002). Therefore, it was proposed that the tumor microenvironment may educate TAMs so that they produce important growth factors and enzymes that stimulate angiogenesis and tumor growth (Bingle et al., 2002; Mantovani et al., 2004; Pollard, 2004). More specifically, tumor cells have been suggested to redirect TAM activity by secreting cytokines like IL-10 and TGF-β. Thus, TAMs may have either tumor-suppressing or tumor-promoting functions, depending on their activation state. Our data obtained in a mouse model for myeloma suggest that tumor-specific CD4+ T cells may have a pivotal role in preventing early tumorigenesis by secreting IFNγ and inducing the classical macrophage activation pathway that results in inhibition of tumor cell growth.

Experimental Procedures

Mice, Cell Lines, and Injection of Tumor Cells

Id-specific TCR-transgenic SCID mice or SCID littermates on a BALB/c background (Bogen et al., 1992, 1995) were bred in a heterozygous (nontransgenic SCID × TCR-transgenic SCID) state by Taconic (Ry, Denmark). MOPC315 (IgA, $\lambda 2^{315}$) and J558 (IgA, λ1) are transplantable BALB/c plasmacytomas obtained from American Type Culture Collection (ATCC, Manassas) and propagated as in vitro growing cells. The MOPC-GFP cell line has been described (Dellacasagrande et al., 2003). Tumor cells were washed once in PBS (GIBCO-BRL, Carlsbad, CA) and suspended in PBS prior to s.c. injection in the interscapular region or in the flank. Adult mice were injected with 1–1.6 × 10^5 tumor cells suspended either in 100 μl PBS or in 300 μl Growth Factor Reduced Matrigel (BD Biosciences, San Diego, CA). The study was approved by the National Committee for Animal Experiments (Oslo, Norway).

Antibodies, ELISA, IFNγ

The following commercially available mAbs were used, conjugated with either FITC, PE, allophycocyanin (APC), Alexa 546, or biotin: CD4 (RM4.5 or GK1.5), CD8 (53-6.7), CD11b (M1/70), CD11c (HL3), CD23 (B3B4), CD44 (IM7), CD45R/B220 (RA3-6B2), CD54 (3E2), CD138 (281-2), MHC-II I-A/I-E (M5/114.15.2), TCRCβ (H57-597), Mac-3 (M3/84) (PharMingen, San Diego, CA); CD11a (I21/7), CD62L (MEL-14), CD69 (H1.2F3), CD80 (1G10), CD86 (GL1), MHC-II I-A (NIMR-4), (Southern Biotechnology, Birmingham, AL); CD204 (2F8), CD206 (MR5D3) (Serotec, Oxford, UK); von Willebrand factor (Dako, Glostrup, Denmark); goat anti-mouse IgG (Molecular Probes, Eugene, OR); donkey anti-rat IgG, donkey anti-mouse IgG, donkey anti-rabbit IgG (Jackson ImmunoResearch, CA). The following mAbs were affinity purified and conjugated with FITC or biotin in our laboratory: transgenic-TCR-clonotype (GB113), MHC-II I-E (14-4-4S; ATCC), F4/80 (HB198, ATCC), TCR Vβ8 (F23.1), FcγRII/III (2.4G2; ATCC), IFNγ (XMG1.2), M315 (Ab2.1-4), Ig Cα (8D2), Ig κ-chain (187.1). M315 myeloma protein in serum was quantified by ELISA (Lauritzsen and Bogen, 1993). Mouse recombinant IFNγ was a kind gift from Dr. G. Garotta (Hoffmann-La Roche, Basel, Switzerland).

Analysis of Cells by Flow Cytometry

Matrigel plugs were treated with collagenase and DNase (both from Sigma, St. Louis) at 37°C for 30 min to release the cells. Single-cell suspensions from draining (axillaris + inguinal) LN were made by squeezing LN through a stainless steel sieve (Sigma). Unspecific binding was blocked by incubation with heat-inactivated 30% normal rat serum in PBS and 100 μg/ml anti-FcγRII/III mAb (2.4G2) prior to staining with specific mAbs. Biotinylated mAbs were detected with streptavidin conjugated to peridinin chlorophyll protein (PerCP, PharMingen). For measurement of DNA synthesis, mice were treated with BrdU i.p. (2 × 1 mg on day +3 and +7 after tumor cell injection), and it was added to their drinking water (0.8 mg/ml, starting on day +3), before staining at day +9 with APC BrdU flow kit (BD Biosciences). For intracellular cytokine detection, cells were stimulated with the phorbol myristate acetate and ionomycin (both from Sigma) in vitro for 6 hr in cell culture medium supplemented with monensin prior to staining with Cytofix/Cytosperm Plus reagents (PharMingen) and specific mAbs. Quadruple-stained cells were analyzed on a FACScalibur instrument with CELLQUEST software (BD Biosciences).

Immunohistochemistry

Upon termination of the experiment, the Matrigel plugs were removed by a wide excision of the flank wall, including the skin and all muscle layers, and transferred to PBS on ice. Each specimen was then divided into two pieces, one being immediately snap frozen (liquid N_2) in OCT compound (Sakura, Torrance, CA) for storage at −70°C until use, the other being fixed for 24 hr in methanol (−20°C) and further processed as follows: 96% ethanol (24 hr, 4°C), absolute ethanol (2 hr, 4°C) twice, xylene twice (20 hr, 4°C and 30 min, 22°C) prior to paraffin embedding. All of the following steps were performed at 22°C unless otherwise noted. Frozen tissue sections (8 μm) were air dried overnight and subsequently acetone-fixed. Paraffin sections (4 μm) were dried for 24 hr at 4°C, dewaxed with xylene, and bathed consecutively in absolute ethanol, 96% ethanol, 70% ethanol, and PBS before staining. Cryosections were incubated with the primary and secondary reagents for 1 hr each, whereas paraffin sections were incubated with primary antibodies for 20 hr and secondary reagents for 3 hr, repsectively. In some experiments Hoechst blue nuclear stain was added to the final volume of washing buffer after the last incubation. Negative controls were tissue sections incubated with primary irrelevant isotype- and concentration-matched mAbs. Microscopy was performed with a Nikon E-800 fluorescence microscope (Nikon Corp., Tokyo, Japan) equipped with a F-view digital camera controlled by the analySIS 3.2 software (Soft Imaging System GmbH, Munster, Germany).

T Cell Proliferation Assay

Matrigel-infiltrating CD11b+ cells were purified by FACSDiVa (BD Biosciences) at day 5 after s.c. injection. Sorted CD11b+ cells (≥96% pure) were irradiated (2000 rad) and used as stimulators (5 × 10^4 cells per well). Responders (2 × 10^4 cells per well) were short-term cultured Th2-polarized LN cells from TCR-transgenic mice (Munthe et al., 1999). An optimal concentration of the Id 89-107 synthetic peptide (4 μg/ml) was added to the positive control. Cultures were pulsed with [^{3}H]thymidine (Montebello Diagnostics, Oslo, Norway) after 72 hr and harvested 12 hr later on a TopCount NXT microplate counter (Packard, Meriden, CT).

Tumor Cell Growth Inhibition Assay

Matrigel-infiltrating CD11b+ cells were purified by FACSDiVa at day 7 after s.c. injection. Sorted CD11b+ cells (≥98% pure) were irradiated (2000 rad) and added at various effector:target ratios to MOPC315 cell cultures (10^4 tumor cells/well). Alternatively, MOPC315 cells were cultured in the presence of 5000 U/ml mouse recombinant IFNγ. Cultures were pulsed with [^{3}H]thymidine after 48 hr and harvested 12 hr later on a TopCount NXT microplate counter.

Supplemental Data

Supplemental Data including an Experimental Procedures section detailing immunohistochemistry and two figures are available with

this article online at http://www.immunity.com/cgi/content/full/22/3/371/DC1/.

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