Interleukin-1 is required for cancer eradication mediated by tumor-specific Th1 cells

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Abbreviations: APC, antigen-presenting cell; ATCC, American Type Culture Collection; FGF-b, fibroblast growth factor-b; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; ICAM-1, intercellular adhesion molecule-1; IFN-γ, interferon-γ; Ig, immunoglobulin; IL, interleukin; IL-1Ra, IL-1 receptor antagonist; i.p., intraperitoneally; LIF, leukemia inhibitory factor; LN, lymph node; LPS, lipopolysaccharide; mAb, monoclonal antibody; M-CSF, monocyte colony-stimulating factor; MHC, major histocompatibility complex; PBS, phosphate-buffered saline; PDGF-bb, platelet-derived growth factor-bb; s.c., subcutaneously; SCID, severe combined immunodeficiency; TCR, T cell receptor; Tg, transgenic; TGF-β, transforming growth factor-β; Th, T helper; TNFα, tumor necrosis factor α; TRAIL, TNF-related apoptosis-inducing ligand; TWEAK, TNF-related weak inducer of apoptosis.

The role of inflammation in cancer is controversial as both tumor-promoting and tumor-suppressive aspects of inflammation have been reported. In particular, it has been shown that pro-inflammatory cytokines, like interleukin-1α (IL-1α), IL-1β, IL-6, and tumor necrosis factor α (TNFα), may either promote or suppress cancer. However, the cellular and molecular basis underlying these opposing outcomes remains enigmatic. Using mouse models for myeloma and lymphoma, we have recently reported that inflammation driven by tumor-specific T helper 1 (Th1) cells conferred protection against B-cell cancer and that interferon-γ (IFN-γ) was essential for this process. Here, we have investigated the contribution of several inflammatory mediators. Myeloma eradication by Th1 cells was not affected by inhibition of TNFα, TNF-related weak inducer of apoptosis (TWEAK), or TNF-related apoptosis-inducing ligand (TRAIL). In contrast, cancer elimination by tumor-specific Th1 cells was severely impaired by the in vivo neutralization of both IL-1α and IL-1β (collectively named IL-1) with IL-1 receptor antagonist (IL-1Ra). The antitumor functions of tumor-specific Th1 cells and tumor-infiltrating macrophages were both affected by IL-1 neutralization. Secretion of the Th1-derived cytokines IL-2 and IFN-γ at the incipient tumor site was severely reduced by IL-1 blockade. Moreover, IL-1 was shown to synergize with IFN-γ for induction of tumoricidal activity in tumor-infiltrating macrophages. This synergy between IL-1 and IFN-γ may explain how inflammation, when driven by tumor-specific Th1 cells, represses rather than promotes cancer. Collectively, the data reveal a central role of inflammation, and more specifically of the canonical pro-inflammatory cytokine IL-1, in enhancing Th1-mediated immunity against cancer.

Introduction

There is an ongoing debate concerning the role of inflammation in cancer because both tumor-promoting and tumor-suppressive aspects of inflammation have been reported. Tumor-specific Th1 cells, which produce the cytokine interferon-γ (IFN-γ), seem to be particularly efficient at fighting cancer.1-6 Accordingly, we and others have proposed that inflammation driven by tumor-specific Th1 cells, with resultant IFN-γ-mediated activation of tumor-infiltrating macrophages, may suppress malignancies.7-9 In contrast, Th2 cells, alternatively activated macrophages (often called “M2 macrophages”), and chronic inflammation may instead promote tumor growth and metastasis.8-13 Thus, the opposing effects of inflammation in various cancer situations may reflect the existence of various types of inflammations.9 However, it remains largely unclear how the same inflammatory mediators, such as cytokines, may either promote or suppress cancer in various contexts.

To characterize a protective inflammatory immune response mediated by tumor-specific Th1 cells, we have used myeloma-
specific T cell receptor transgenic (TCR-Tg) mice. In this transgenic system, CD4\(^+\) T cells recognize a bona fide tumor-specific antigen (idiotype) derived from the L-chain variable region of the immunoglobulin A (IgA) that is secreted by the MOPC315 murine myeloma cell line.\(^{14,15}\) Because, MOPC315 cells lack major histocompatibility complex (MHC) class II molecules, recognition of MOPC315 by CD4\(^+\) T cells occurs via indirect presentation of secreted antigens on tumor-infiltrating antigen-presenting cells (APCs), predominantly macrophages.\(^1\) Idiotyp-specific TCR-Tg mice were made homozygous for the severe combined immunodeficiency (SCID) mutation, which prevents rearrangement of endogenous TCR chains and thereby ensures the unique specificity of the T cells.\(^{16}\) The high frequency of tumor-specific CD4\(^+\) T cells in TCR-Tg SCID mice renders the mice resistant to subcutaneously (s.c.) injected syngeneic MOPC315 myeloma cells, while non-transgenic mice rapidly develop tumors. In this model system, cancer prevention by the immune system (i.e. cancer immunosurveillance) is antigen-specific, depends on antigen secretion by the cancer cells, is mediated by CD4\(^+\) T cells, and does not require the presence of B cells, γδ T cells, CD8\(^+\) T cells, or natural killer (NK) cells.\(^{15-18}\)

In a series of reports, we have described the main cellular events leading to MOPC315 myeloma clearance in idiotyp-specific TCR-Tg SCID mice.\(^1,17-20\) Starting at around day +3 after the s.c. injection of MOPC315 cells, naive tumor-specific CD4\(^+\) T cells become activated in the lymph node (LN) that drains the incipient tumor site.\(^1\) In this draining LN, tumor-specific CD4\(^+\) T cells acquire a Th1 phenotype characterized by the production of IFN\(\gamma\).\(^{1,20}\) Effector tumor-specific Th1 cells migrate from the draining LN to the incipient tumor site, where they collaborate with macrophages in order to recognize and eliminate myeloma cells.\(^1,19,20\) Upon recognition of tumor-derived idiotypic peptides presented on MHC class II molecules by macrophages, tumor-specific Th1 cells secrete IFN\(\gamma\). IFN\(\gamma\) is required for successful myeloma eradication and a dual role of IFN\(\gamma\) was identified. First, IFN\(\gamma\) renders macrophages cytotoxic to myeloma cells. Second, IFN\(\gamma\) induces macrophages to secrete the angiostatic chemokines CXCL9 and CXCL10, which may halt tumor progression by inhibiting angiogenesis.\(^1,18\) The peak of the antitumor immune response is at around day +8 after MOPC315 injection, when the strongest activation of CD4\(^+\) T cells and macrophages is observed.\(^1,20\) Most myeloma cells are eradicated by day +15 after s.c. injection into idiotyp-specific TCR-Tg SCID mice.\(^1\) We have recently reported that myeloma eradication by idiotyp-specific TCR-Tg SCID mice was associated with increased levels, at the incipient tumor site, of several canonical pro-inflammatory cytokines including interleukin (IL)-1\(\alpha\), IL-1\(\beta\), IL-6, and tumor necrosis factor α (TNF\(\alpha\)).\(^{1,18}\) We concluded that inflammation driven by tumor-specific Th1 cells protects against B-cell cancer.\(^{1,18}\) However, the importance of the inflammatory reaction itself for cancer eradication was not investigated.

In the present study, the importance of inflammation for cancer eradication by tumor-specific Th1 cells was evaluated by selectively blocking several inflammatory mediators. We report that successful myeloma rejection by TCR-Tg SCID mice required neither TNF\(\alpha\), TWEAK, nor TRAIL. In contrast, tumor elimination was severely impaired by in vivo neutralization of both IL-1\(\alpha\) and IL-1\(\beta\) (collectively named IL-1) with IL-1 receptor antagonist (IL-1Ra). IL-1 was shown to be essential for the antitumor functions of both tumor-specific Th1 cells and tumor-infiltrating macrophages. Collectively, the data reveal the central role of inflammation, and more specifically of the canonical pro-inflammatory cytokine IL-1, in supporting Th1-mediated adaptive immunity against cancer.

**Results**

Myeloma eradication by Th1 cells is associated with sustained macrophage activation and local release of numerous inflammatory cytokines

In order to monitor macrophage activation and cytokine secretion at the incipient tumor site during the course of myeloma eradication by tumor-specific Th1 cells, we used the Matrigel cytokine assay. This technique consists of injecting mice s.c. with myeloma cells embedded in a collagen gel named Matrigel.\(^{1,18}\) At various time points after injection, the Matrigel plugs may be excised, allowing the analysis of infiltrating immune cells and locally secreted cytokines (Fig. 1A). For comparison, two groups of mice were injected: tumor-resistant TCR-Tg SCID mice and tumor-prone non-transgenic SCID mice. In both types of mice, we have previously reported that MOPC315-containing Matrigel plugs were rapidly infiltrated by a large population of CD11b\(^+\) cells, which consisted almost exclusively of macrophages with possibly a few CD11b\(^+\) CD11c\(^+\) dendritic cells.\(^1\) In TCR-Tg SCID mice, we have also shown that Th1-derived IFN-γ induced both upregulation of surface MHC class II molecules and tumoricidal activity in Matrigel-infiltrating macrophages.\(^1,18\) Therefore, we measured surface MHC class II levels on Matrigel-infiltrating CD11b\(^+\) cells in order to assess macrophage activation by Th1 cells at various time points after s.c. injection of MOPC315 cells. In parallel, the levels of 46 cytokines in the Matrigel extracellular matrix were quantified. On day +2 after injection, no difference was observed between the groups, concerning macrophage activation and cytokine levels (data not shown). At day +5, +8, and +11, surface MHC class II levels on Matrigel-infiltrating CD11b\(^+\) macrophages were found to be significantly upregulated in tumor-resistant TCR-Tg SCID mice, compared to tumor-prone SCID mice (Fig. 1B). Moreover, myeloma eradication was accompanied by a significant increase in the local concentration of 29 cytokines at day +5, 23 cytokines at day +8, and 30 cytokines at day +11 (Fig. 1C). In contrast, a few cytokines with known anti-inflammatory properties, like transforming growth factor (TGF)-β1, TGF-β2, TGF-β3, and IL-10, had lower levels in tumor-resistant TCR-Tg SCID compared to tumor-prone SCID mice at day +8 and day +11 (Fig. 1C). Thus, successful myeloma eradication mediated by tumor-specific Th1 cells was associated with sustained IFN-γ-mediated activation of macrophages and with local release of numerous inflammatory mediators including IL-1\(\alpha\), IL-1\(\beta\), IL-6, IL-23, and TNF\(\alpha\).
IL-1 is required for myeloma elimination by tumor-specific Th1 cells

To clarify the mechanism of myeloma eradication, we performed a series of tumor challenge experiments, using established protocols to selectively block candidate effector molecules. As previously reported, tumor rejection by TCR-Tg SCID mice was abrogated by blocking IFN-γ in vivo with a monoclonal antibody (mAb) (Fig. 2A). In contrast, no effect was observed when the mice were treated with blocking mAb to neutralize TNFα, TWEAK, or TRAIL, suggesting that these molecules were not
essential for myeloma elimination by Th1 cells (Figs. 2B–D). Strikingly, neutralization of the common receptor for both IL-1α and IL-1β with IL-1Ra severely impaired tumor protection in TCR-Tg mice (Fig. 2E). Moreover, when using serum concentration of the myeloma protein (the monoclonal IgA secreted by MOPC315) as a surrogate marker of tumor burden, mice treated with IL-1Ra had significantly higher levels of myeloma protein as compared to vehicle-treated mice at day +17 (Supplementary Fig. S1). Thus, protective antitumor immunity in TCR-Tg SCID mice is dependent on both IFN-γ and IL-1.

IL-1 is required for the antitumor functions of both tumor-specific Th1 cells and tumor-infiltrating macrophages

The role of IL-1 for myeloma eradication by Th1 cells was investigated by treating tumor-specific TCR-Tg SCID mice with IL-1Ra (or vehicle) before analyzing the immune response at day +8 (Fig. 3A). Neutralization of both IL-1α and IL-1β with IL-1Ra had no clear effect on the activation of naïve tumor-specific CD4+ T cells in the LN draining the incipient tumor site, as measured by expression of the early activation marker CD69 (Fig. 3B). Moreover, intracellular staining of IFN-γ and T-bet indicated that IL-1 blockade did not influence the Th1 polarization of the tumor-specific CD4+ T cells in LN and Matrigel plugs (Fig. 3B). However, quantification of 46 locally secreted cytokines revealed a significant downregulation of 11 cytokines (soluble CD40L, G-CSF, GM-CSF, IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-17A, and IL-25) and a significant upregulation of two molecules (soluble ICAM-1 and TGF-β1) in the IL-1Ra-treated group (Fig. 3C). The significant reduction of two critical Th1-derived cytokines, namely IFN-γ and IL-2, in the IL-1Ra-treated group strongly suggests that IL-1 is regulating...
the function (i.e., cytokine secretion) of tumor-specific Th1 cells at the incipient tumor site.

The effects of IL-1 neutralization on tumor-infiltrating macrophages were also investigated. Matrigel-infiltrating CD11b+ macrophages were significantly less activated as measured by surface MHC class II levels in mice that had received IL-1Ra when compared to controls (Fig. 3D). In the next experiment, we tested whether this reduction in macrophage activation upon IL-1Ra treatment was associated with lower tumoricidal activity. Matrigel-infiltrating CD11b+ macrophages from the two experimental groups were tested for their ability to inhibit MOPC315 myeloma growth in vitro. In accordance with previous reports,1,17,18 sorted Matrigel-infiltrating macrophages from control NaCl-treated TCR-Tg SCID mice strongly suppressed the in vitro growth of MOPC315 myeloma cells (Fig. 3E, 70 percent myeloma growth inhibition at 20:1 effector to target ratio). In contrast, macrophages isolated from IL-1Ra-treated mice were essentially unable to suppress MOPC315 in vitro growth, even at the highest (20:1) effector to target ratio (Fig. 3E, only 20% residual growth inhibition). Collectively, the data reveal that IL-1 has a central role in the antitumor effector functions of both tumor-specific Th1 cells (cytokine secretion) and tumor-infiltrating macrophages (activation and tumoricidal activity).

IFN-γ and IL-1β synergize to induce tumoricidal activity in macrophages

The effect of IL-1 on macrophage activation was further investigated in vitro using non-activated macrophages isolated from non-transgenic SCID mice. In accordance with a previous report,1 Matrigel-infiltrating macrophages purified from SCID mice were unable to inhibit MOPC315 in vitro growth (Fig. 4). Addition of IFN-γ into the culture medium was not sufficient by itself to induce tumoricidal activity in

Figure 3. Roles of IL-1 for myeloma eradication mediated by tumor-specific Th1 cells. (A) Experimental design. Idiotype-specific TCR-Tg SCID mice were injected s.c. with MOC315 in Matrigel and treated s.c. daily with IL-1Ra or vehicle (NaCl). Draining LN and Matrigel plugs were analyzed at day +8. (B) Tumor-specific CD4+ T cells in pooled draining LN and Matrigel plugs from IL-1Ra or NaCl-treated mice (n = 8–10 mice per group) were analyzed by flow cytometry. Tumor-specific T cells were gated using the GB113 mAb specific for the transgenic TCR. Levels of surface CD69 or intracellular T-bet and IFN-γ were recorded (solid lines). Dotted lines indicate isotype-matched control mAb. (C) The concentration of 46 cytokines in the extracellular matrix of the Matrigel plugs was quantified for IL-1Ra-treated (black bars) and NaCl-treated (gray bars) TCR-Tg SCID mice (n = 11, mean ± SD). Only cytokines which showed significantly (p < 0.05, Mann-Whitney test) higher levels in one group compared with the other are included in the bar graphs. (D) MHC class II levels on Matrigel-infiltrating CD11b+ cells in NaCl or IL-1Ra-treated TCR-Tg SCID mice (n = 8–10, mean ± SD). **p = 0.003 (Mann-Whitney test). (E) Growth inhibition assay. Matrigel-infiltrating CD11b+ macrophages were isolated from IL-1Ra (black bars) or NaCl (gray bars)-treated TCR-Tg SCID mice (n = 10 mice per group), pooled for each group and tested, at various effector to target ratios, for their ability to suppress the in vitro proliferation of MOPC315 cells (mean of quadruplicates ±SD). Mø, macrophages.
macrophages. In contrast, when macrophages were treated with IL-1Ra, which blocks both IL-1α and IL-1β, revealed that IL-1 was essential for tumor elimination by Th1 cells. The partial observed effect for IL-1Ra is presumably due to incomplete receptor blockade because of the very short half-life (<2 h in serum) that has been reported for IL-1Ra in vivo.28 Thus, both IFN-γ and IL-1 are required for successful antitumor immunity mediated by tumor-specific Th1 cells. Our study reveals a central role of inflammation, and more specifically of the canonical pro-inflammatory cytokine IL-1, in enhancing Th1-mediated immunity against cancer.

To our knowledge, our data represent the first direct demonstration of the central role of IL-1 for successful antitumor immunity. There are, however, a few previous reports in mice suggesting that IL-1 may be used therapeutically to treat cancer. Intratumoral injection of IL-1α successfully cured mice from MethA sarcoma and B16 melanoma.29 Moreover, IL-1α injected intramuscularly was effective in reducing the number of lung metastases in mice with Lewis lung carcinoma.29 Intraperitoneal injection of IL-1β on days +6–8 of tumor growth caused complete regression of subcutaneous SA1 sarcoma and L5178Y lymphoma in mice.30 Experiments with T-cell deficient mice and cell transfer suggested that the therapeutic effect of IL-1β consisted in stimulating the antitumor immunity mediated by CD4+ T cells.30 Activated invasive RO1 T-lymphoma cells that displayed short-term IL-1α expression manifested reduced tumorigenicity and could be used to treat mice with lymphoma.31 Similarly, fibrosarcoma cells transfected with IL-1α became strongly immunogenic and failed to generate tumors in mice.32 It was proposed that IL-1α was activating the antitumor immune response mediated by CD8+ T cells and NK cells.32 In summary, our data represent the first demonstration that IL-1 is required for successful antitumor immunity. However, the antitumor therapeutic potential of IL-1 has been suggested by several mouse studies and a role for CD4+ T cells, CD8+ T cells, and NK cells has been proposed.

We performed a series of experiments to clarify the role if IL-1 in antitumor immunity. IL-1 neutralization was shown to affect the antitumor functions of both tumor-specific Th1 cells and tumor-infiltrating macrophages. IL-1 had a central role in promoting a cancer-suppressive pro-inflammatory cytokine milieu at the incipient tumor site. In particular, secretion of the Th1-derived cytokines IL-2 and IFN-γ at the incipient tumor site was severely reduced by IL-1 blockade. Our study revealing the central role of IL-1 for T-cell mediated antitumor immunity are in accordance with published data suggesting a key function of IL-1 for T-cell biology in general, in particular for Th1 cells. For example, IL-1α has been reported to promote Th1 differentiation allowing successful immunity against the parasite Leishmania major.33 Experiments with model antigens such as pigeon cytochrome C and ovalbumin revealed the importance of IL-1 for CD4+ T-cell expansion and survival.34 An early study by Ralph Steinman and coworkers revealed that IL-1 could enhance T-cell dependent immunity by amplifying the function of dendritic cells.35 More recently, IL-1 was shown to enhance the expansion and effector functions of CD8+ T cells that were specific for various antigens such as ovalbumin or pathogen-derived peptides.36 Collectively, available data support a key role of the pro-
inflammatory cytokines IL-1α and IL-β for T-cell mediated immunity against both pathogens and cancer.

We here report that in vivo blockade of IL-1α and IL-1β with IL-1Ra impaired the activation and tumoricidal activity of tumor-infiltrating macrophages. Moreover, IL-1 was shown in vitro to synergize with IFN-γ for induction of tumoricidal activity in macrophages. Previous in vitro studies revealed that two signals are required to render macrophages cytotoxic to cancer cells. IFN-γ was the first cytokine clearly identified to activate macrophages to become tumoricidal, when given in combination with dead bacteria or bacterial products such as LPS. It is now established that LPS activation of macrophages through toll-like receptor-4 (TLR4) ligation and signaling, as the IL-1R and TLR-4 share a common intracellular signaling pathway, it is not unexpected that IL-1 may replace LPS for rendering macrophages tumoricidal. In fact, an in vitro study by Hori et al. showed that combined use of IFN-γ and IL-1 could render macrophages tumoricidal against TNFα-insensitive P815 murine mastocytoma cells, in a concentration dependent manner. This is consistent with our findings, showing that tumor-infiltrating macrophages that do not demonstrate an antitumor activity can become tumoricidal when treated with a combination of IFN-γ and optimal concentrations of IL-1β. The observation that high concentrations of IL-1β failed to activate macrophages, even in the presence of IFN-γ, indicates a negative feedback loop in the induction of macrophages tumoricidal activity. Niinobu and colleagues reported that high-levels of nitric oxide released from cytokine-activated macrophages (IFN-γ, TNF-α, and IL-1β) may sensitize macrophages themselves to Fas-mediated apoptosis. They further hypothesized that this mechanism may be a negative feedback loop serving to promote resolution of inflammation by accelerating deletion of macrophages by apoptosis. Thus, our data on the induction of macrophage tumoricidal activity by optimal concentrations of IL-1 in conjunction with IFN-γ confirm previous in vitro observations and validate these findings in vivo using a mouse model for cancer eradication mediated by Th1-activated macrophages.

In a previous study with the same model system, we measured 33 cytokines at the incipient tumor site at one single time point, namely day +8, which represents the peak of the antitumor immune response. We have now expanded this work by including several additional time points (day +2, +5, and +11) and 14 new cytokines (CCL11, CCL20, soluble CD40L, soluble ICAM-1, IL-17F, IL-21, IL-22, IL-23p19, IL-25, IL-27p28, IL-31, IL33, TGF-B2, TGF-β3). The kinetics data revealed that the increased levels of inflammatory cytokines (such as IL-1, IL-6, and TNF-α) and the activation of tumor-infiltrating macrophages were not restricted to day +8 but were observed during the whole process of myeloma rejection, from day +5 until day +11. All the new cytokines investigated were found to be significantly either upregulated (CCL11, CCL20, soluble CD40L, IL-17F, IL-21, IL-22, IL-23p19, IL-25, IL-27p28, IL-31, and IL33) or downregulated (soluble ICAM-1, TGF-B2, and TGF-β3) at one or several time points. Several of these cytokines associated with cancer rejection should deserve further investigations. In particular, IL-21, IL-27, and IL33, that were all upregulated at three time points investigated (day +5, +8, and +11) may be particularly promising candidates for cancer immunotherapy. In fact, IL-21 has already been used to treat patients with metastatic melanoma and renal cell carcinoma in phase I/II trials and promising antitumor activities were reported. IL-27 was shown to be able to inhibit the growth of human multiple myeloma and acute myeloid leukemia cells injected into immunodeficient mice. Moreover, the alarmin IL-33 has been reported, in mouse models, to enhance antitumor immune responses mediated by Th1 cells, CD8+ T cells, and NK cells.

The role of inflammation and inflammatory cytokines in cancer is controversial. On one hand, inflammation is widely considered to be detrimental in terms of cancer occurrence, growth and metastasis. For instance, it is well documented that chronic inflammatory diseases such as ulcerative colitis, gastritis, and rheumatoid arthritis lead to increased risk of developing colorectal cancer, gastric cancer, and lymphoma, respectively. High IL-6 serum levels were reported to be associated with shorter survival of patients with B-cell malignancies or lung cancer. Studies in mice have documented tumor-promoting effects of IL-1α and IL-1β, in particular for cancer metastasis and tumor angiogenesis. Furthermore, epidemiological studies indicated that daily intake of aspirin, a non-steroidal anti-inflammatory drug, was associated with reduced cancer risk. On the basis of these observations, dampening inflammation has been suggested as a novel strategy to fight cancer. Our mouse data suggest that IL-1 blockade may weaken T-cell mediated antitumor immunity and thereby may potentially have a detrimental effect on patients with cancer.

Cancer-suppressive aspects of inflammation are also well documented (reviewed in). For example, high densities of tumor-infiltrating immune cells, which is a typical sign of inflammation, have been shown to be associated with longer patient survival for several malignancies including ovarian, colorectal, and breast cancers. Certain types of cancer chemotherapies appear to stimulate antitumor immunity and mouse studies revealed a key role of IL-1β in this process. Therefore, considering inflammation as a cancer-promoting process only is likely to be a misleading oversimplification. To solve the controversy, we have proposed that the opposing effects of inflammation in various cancer situations may reflect the existence of various types of inflammations. On the basis of our mouse studies with the MOPC315 myeloma, we have previously reported that successful cancer immunosurveillance mediated by tumor-specific CD4+ T cells was consistently associated with elevated local levels of both pro-inflammatory (IL-1α, IL-1β, IL-6) and Th1-associated cytokines (IFNγ, IL-2 and IL-12). Cancer eradication was achieved by collaboration between tumor-specific Th1 cells and tumor-infiltrating, antigen-presenting macrophages. Th1 cells induced secretion of IL-1β and IL-6 by macrophages. Th1-derived IFNγ was shown to render macrophages directly cytotoxic to cancer cells, and to induce macrophages to secrete angiostatic chemokines. From this previous work, we concluded that inflammation, when
Th1-mediated antitumor immunity. Cancer by revealing the central role of IL-1 in enhancing a beneficial function of certain types of inflammations against cancer was protected against cancer.

Our present study provides direct pieces of evidence for a beneficial function of certain types of inflammations against cancer by revealing the central role of IL-1 in enhancing Th1-mediated antitumor immunity.

Materials and Methods

Mice and injection of tumor cells

Heterozygous idiotype (2.315)-specific TCR-Tg SCID mice or SCID littersmates on BALB/c background were bred and housed at the Department of Comparative Medicine, Oslo University Hospital Rikshospitalet, Oslo, Norway. MOPC315 is a transplantable BALB/c plasmacytoma obtained from the American Type Culture Collection (ATCC) and propagated as in vitro growing cells. Adult mice were injected s.c., in the interscapular region or in the flank, with 1–1.6 × 10³ MOPC315 cells subcutaneously. Tumor growth was followed over time by palpation. Mice with a tumor diameter ≥10 mm were euthanized. The study was approved by the National Committee for Animal Experiments (Oslo, Norway).

Matrigel cytokine assay

A detailed protocol for the Matrigel cytokine assay is available at www.nature.com/protocolexchange/protocols/2130. Briefly, MOPC315 cells were mixed with ice-cold Matrigel and 250 μL of the mixture was carefully injected s.c. into each mouse under anesthesia. Matrigel, which is liquid at +4°C, gellifies at body temperature and forms a gel plug containing the cancer cells. At various time points after injection, mice were sacrificed and the s.c. Matrigel plugs were excised and treated with collagenase. Dissolved Matrigel solution was centrifuged and the cell pellet was analyzed by flow cytometry. Cytokine levels in Matrigel supernatants were measured by Luminex technology using single-plex or multiplex bead assays (Bio-Plex, Bio-Rad Laboratories). Samples were analyzed as singlets or duplicates, and standards in duplicates, using a Luminex-100 instrument with Bio-Plex Manager 6.0 software (Bio-Rad Laboratories). The following 46 cytokines were measured: CCL2, CCL3, CCL4, CCL5, CCL11, CCL20, soluble CD40L, CXCL1, CXCL2, CXCL9, FGF-basic, G-CSF, GM-CSF, soluble ICAM-1, IFN-γ, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p40, IL-12p70, IL-13, IL-15, IL-17A, IL-17F, IL-18, IL-21, IL-22, IL-23p19, IL-25, IL-27p28, IL-31, IL-33, IFN-α, IFN-β, M-CSF, PDGF-bb, TGF-α, TGF-β, TNFα, and VEGF.

Analysis of cells by flow cytometry

Single-cell suspensions from draining axillary LN and Matrigel plugs were obtained by use of a stainless steel sieve (Sigma). Non-specific antibody binding was blocked by incubation with PBS containing 30% heat-inactivated (56°C, 30 min) normal rat serum and 100 μg/mL anti-FcγRI/III mAb (clone 2.4G2, ATCC). Cells were stained for 15 min on ice with specific mAb in PBS supplemented with 0.5% bovine serum albumin (Bovest). The following commercially available mAb were used, conjugated with fluorescein, phycoerythrin, allophycocyanin or biotin: CD4+ (GK1.5), CD11b (M1/70), IFNγ (XMG1.2), MHC class II A-I-E (M5/114.15.2), TCR β chain (H57-597) (BD Biosciences); CD11b (3A33), CD69 (H1.2F3) (Southern Biotechnology); T-bet (4B10) (BioScience). GB113 is a clonotype-specific mAb which recognizes the λ2315-specific TCR expressed by the TCR-Tg SCID mice. GB113 and 2.4G2 (anti-FcyRII/III) mAb were affinity-purified, and GB113 was further biotinylated in our laboratory. Biotinylated mAb were detected with streptavidin conjugated to peridinin chlorophyll protein (BD Biosciences). For intracellular cytokine detection, cells were stimulated with phorbol myristate acetate and ionomycin (both from Sigma) in the presence of monensin (GolgiStop, BD Biosciences) in vitro for 4 h before staining with Cytofix/ Cytoperm Plus reagents (BD Biosciences) and specific mAb. Quadruple-stained cells were analyzed on a FACSscalibur instrument with CellQuestPro (BD Biosciences) and FlowJo version 10 (FlowJo) softwares.

In vivo blockade of IFN-γ, TNFα, TWEAK, and TRAIL

In accordance with previously established protocols, mice were injected intraperitoneally (i.p.) three times a week with 250 μg blocking mAb against IFN-γ (XMG2.1, ATCC), TNFα (μP6-XT22), TWEAK (MTW-1), or TRAIL (N2B2), or with isotype-matched control rat IgG1 (Y13-259, ATCC) or rat IgG2a (Y13-238, ATCC) mAb. Neutralization of the common receptor for IL-1α and IL-1β with IL-1Ra

For tumor challenge experiments (Fig. 2), Alzet osmotic pumps (model 2004; 0.25 μL/h, 28 d) were filled with 200 μL of 6.7 mg/mL IL-1Ra (Anakinra/Kineret, Biovitrum) or vehicle (9 mg/mL NaCl) and implanted s.c. on the flank of anesthetized mice. MOPC315 myeloma cells (1.6 × 10⁵) suspended in 100 μL PBS were injected s.c. where the delivery tip of the osmotic pump was situated. For short-term in vivo experiments (Fig. 3), mice were treated s.c. daily with 100 μL of 400 μg/mL IL-1Ra or vehicle (9 mg/mL NaCl).

Growth inhibition assay with in vivo activated macrophages

TCR-Tg SCID mice (n = 10) were injected s.c. with MOC315 in Matrigel and treated s.c. daily with IL-1Ra or vehicle as described above. Matrigel-infiltrating CD11b+ cells were isolated by FACSaria (BD Biosciences) at day +8. Sorted CD11b+ cells (≥95% pure) were pooled for each group, irradiated (2,000 rad) and added at various effector/target ratios to MOPC315 cell cultures (10⁴ tumor cells per well) in quadruplicates. Cultures were pulsed with [³H]thymidine (Hartmann Analytic) after 48 h and harvested 12 h later. [³H]thymidine was measured on a 1450 MicroBeta Trilux microplate scintillation counter (Perkin Elmer).
Growth inhibition assay with in vitro activated macrophages

SCID mice (n = 12) were injected with MOPC315 in Matrigel. At day +8, CD11b+ cells were isolated using mAb-conjugated magnetic beads (MACS, Miltenyi Biotec). Isolated cells were counted and seeded (10⁵ cells per well) in triplicates in 96-well flat-bottom cell culture plates (Costar). After 2 h incubation at 37°C in 5% CO₂, the cells were washed three times with fresh media (RPMI1640, Gibco), before addition of IFN-γ (GraphPad Software).

Statistical analysis

The cytokine levels were analyzed using the Mann–Whitney test. Statistical analysis was performed utilizing the log-rank test. Flow cytometry data and growth inhibition assay with SCID mice (Legatet til Henrik Homans Minde, and Legatet til mine om H. G. og Andrine Berg og deres søn Hans Gysler Berg).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher’s website.

Author Contributions

O.A.W.H., A.C., and K.B.L. performed the experiments, collected the data, and prepared the figures. H.Y. provided key reagents (mAbs). All authors analyzed and discussed the data. O.A.W.H. and A.C. designed the study and wrote the manuscript. K.B.L., H.Y., and B.B. contributed in writing the manuscript.
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