Tumoral Immune Suppression by Macrophages Expressing Fibroblast Activation Protein-α and Heme Oxygenase-1

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Abstract

The depletion of tumor stromal cells that are marked by their expression of the membrane protein fibroblast activation protein-α (FAP) overcomes immune suppression and allows an anticancer cell immune response to control tumor growth. In subcutaneous tumors established with immunogenic Lewis lung carcinoma cells expressing ovalbumin (LL2/OVA), the FAP+ population is comprised of CD45+ and CD45− cells. In the present study, we further characterize the tumoral FAP+/CD45+ population as a minor subpopulation of F4/80hi/CCR2+/CD206+ M2 macrophages. Using bone marrow chimeric mice in which the primate diphtheria toxin receptor is restricted either to the FAP+/CD45+ or to the FAP+/CD45− subset, we demonstrate by conditionally depleting each subset that both independently contribute to the immune-suppressive tumor microenvironment. A basis for the function of the FAP+/CD45− subset is shown to be the immune inhibitory enzyme, heme oxygenase-1 (HO-1). The FAP+/CD45+ cells are the major tumoral source of HO-1, and an inhibitor of HO-1, Sn mesoporphyrin, causes the same extent of immune-dependent arrest of LL2/OVA tumor growth as does the depletion of these cells. Because this observation of immune suppression by HO-1 expressed by the FAP+/CD45+ stromal cell is replicated in a transplanted model of pancreatic ductal adenocarcinoma, we conclude that pharmacologically targeting this enzyme may improve cancer immunotherapy. Cancer Immunol Res; 2(2); 1-6. © 2013 AACR.

Introduction

The failure of the immune system to control the growth of immunogenic cancers has been ascribed to two general processes: cancer immunoediting and immune suppression. Immunoediting has been demonstrated in models of autochthonous soft tissue sarcomas induced either by a mutagenic agent, methylcholanthrene (1), or by tissue-specific, Cre/LoxP-regulated expression of oncogenic K-rasG12D and deletion of p53 (2). Tumoral immune suppression has been shown in models of transplanted, ectopic tumors (3), and recently in an autochthonous model of lung adenocarcinoma (4). In relation to immune suppression, progress has been made in the clinic with the introduction of therapeutic antibodies targeting immune checkpoints (5–7). However, as a high frequency of patients do not respond to these therapeutic antibodies, it is appropriate to continue studies of the tumoral stromal cells that have immune-suppressive function, including the cell that is identified by its expression of the membrane dipeptidyl peptidase, fibroblast activation protein-α (FAP; ref. 8).

FAP+ stromal cells were first demonstrated in human adenocarcinomas, and subsequently were found in various nonneoplastic, chronic inflammatory lesions (9, 10). Recently, in a genetically modified mouse model in which FAP+ cells express the primate diphtheria toxin receptor (DTR), the conditional depletion of these cells from an established, immunogenic, transplanted tumor caused its growth arrest. The control of tumor growth induced by depleting FAP+ cells depended on adaptive immunity, but did not involve enhanced priming of the CD8+ T cells, leading to the conclusion that FAP+ stromal cells suppressed the function of effector T cells in the tumor microenvironment (8).

Understanding the means of immune suppression by tumoral FAP+ stromal cells is especially challenging because two subtypes occur, a CD45+ mesenchymal population and a hematopoietic subset that is CD45+/CD11b−/Gr-1− (8). The present study focuses on the FAP+/CD45+ tumoral cells, demonstrating that they are a subset of inflammatory macrophages with an M2 phenotype that mediate immune suppression by their expression of HO-1.

Materials and Methods

Mice

FAP/enhanced GFP (EGFP) bacterial artificial chromosome (BAC) transgenic (Tg) and FAP/DTR BAC Tg mice have previously been described (8). C57BL/6-Ly5.1 (CD45.1) mice,
C57BL/6 Rag2−/−, and C57BL/6 (CD45.2; The Jackson Laboratory) were used as indicated. The use of animals was approved by the Ethical Review Committee at the University of Cambridge and the Home Office, United Kingdom.

**Subcutaneous tumor studies and HO inhibition**

Lewis lung carcinoma (LL2)/Thy1.1, LL2/Thy1.1-ovalbumin (OVA; original line purchased from American Type Culture Collection), and PDA (TB 11381, D. Tuveson, CRUK Cambridge Institute) were injected into mice, and the subsequent tumors measured as previously described (8). Sn (IV) mesoporphyrin IX dichloride (SnMP; Frontier Scientific) was dissolved in 0.1 mol/L NaOH and diluted using 0.1 mol/L NaHCO3, pH7. For blocking IFN-γ and TNF-α, mice were injected intraperitoneally at day −1 and 0, relative to SnMP administration, with 12.5 μg/g anti-IFN-γ (XMG1.2) and 10 μg/g anti-TNF-α (MP6-XT3) or 22.5 μg/g nonimmune IgG (eBGR1; eBioscience). Tumor tissue was enzyme-digested to release single cells as previously described (8).

**Flow cytometry**

Antibodies were purchased from eBioscience unless otherwise stated, the following antibodies were used: CCR2 (R&D Systems), CD3 (145-2C11), CD4 (RM4-5), CD8α (eBioH35-17-2), CD11b (M1/70), CD11c (N418), CD14 (Sa2-8), CD16/32 (93), CD31 (390), CD45 (30-F11), CD45.1 (Biolegend, A20), CD45.2 (Biolegend, 104), CD69 (H12F3), F4/80 (BM8), Gr-1 (RB6-8C5), LAMP-1 (eBio1D4B), MHCIId (M5/114.15), and Thy1.2 (53-2.1). Anti-mannose receptor (CD206; R&D Systems) was fluorescently conjugated using the Alexa Fluor 488 antibody labeling kit (Invitrogen) before use. Fc receptors were blocked using anti-CD16/32 (BD Bioscience, 2.4G2). Foxp3 Foxp3 staining was confirmed using the Mouse Regulatory T Cell Staining Kit (eBioscience) before use. Fc receptors were blocked using the Mouse Regulatory T Cell Staining Kit (eBioscience) according to the manufacturer’s protocol. FAP+ cells were stained as previously described (8). Tissue factor-expressing cells were stained with goat anti-tissue factor (R&D Systems) and stained as previously described (8). Tissue factor-positive cells were subjected to flow cytometry analysis after staining with the Mouse Regulatory T Cell Staining Kit (eBioscience) according to the manufacturer’s protocol. FAP+ cells were stained as previously described (8). Dead cells were excluded using 7-amino actinomycin D (Calbiochem). Data were collected using a BD LSR II cytometer and analyzed using FlowJo software (Treestar Inc.).

**Bone marrow chimeras**

Bone marrow cells were flushed from the femurs and tibias of male FAP/DTR BAC Tg (CD45.2; The Jackson Laboratory) mice and stained for FAP as previously described in PBS, 1% fetal calf serum (8). The streptavidin was phycoerythrin (PE)-conjugated (eBioscience). Bone marrow cells were depleted of FAP-expressing cells using anti-PE Microbeads (Miltenyi Bioconjugated (eBioscience). Bone marrow cells were depleted of FAP-expressing cells using anti-PE Microbeads (Miltenyi BioTech) on LD columns according to manufacturer’s protocol to prevent transfer of any donor FAP+ /CD45+ cells present in the bone marrow. FAP-depleted bone marrow cells (10 × 106) were transferred via tail vein injection into recipient female mice, which had been lethally irradiated using a Cesium-137 source irradiator with 2 doses of 6 Gy, 3 hours apart. After 16 weeks, when the bone marrow had reconstituted, LL2/OVA cells were injected. FAP+ cells expressing the FAP/DTR BAC transgene were ablated through the administration of DTX (List Biologicals) at 25 ng/g/24 hours via intraperitoneal injection.

**Immunofluorescence**

Frozen optimum cutting temperature–embedded tumor tissue, which were grown in FAP/EGFP BAC Tg mice, were sectioned and stained as previously described for the preservation of native GFP fluorescence (8). The following antibodies were used: rat anti-F4/80 (AbD Serotec, C3A3-1), rabbit anti-HO-1 (Insight Biotechnology, EP1391Y) and these were detected using NL637 goat anti-rat IgG (R&D Systems) and Alexa Fluor-546 donkey anti-rabbit IgG (Invitrogen). Nuclei were stained using 4′,6-diamidino-2-phenylindole (DAPI; Invitrogen). Images were acquired using a Leica SP2 confocal microscope and associated LCS analysis software.

**qRT-PCR analysis**

mRNA from tumor tissue and sorted cells was extracted using the Absolutely RNA Miniprep Kit (Agilent Technologies). mRNA from sorted populations was amplified using the Trans-polyA Complete whole transcriptome amplification kit (Sigma-Aldrich), and subsequent cDNA purified using a PCR Purification Kit (Qiagen) according to the manufacturer’s protocols. qRT-PCR was performed as previously described using the SuperScript III Platinum One-step qRT-PCR system (Invitrogen; ref. 8). The following primer/probes (Applied Biosystems) were used; HO-1 mm00516006_m1, HO-2 mm00468922_m1, IFN-γ mm99999071_m1, Tbp mm01277045_m1, TNF-α mm99999068_m1.

**Statistical analyses**

Statistical analysis of tumor growth rates was performed using the "CompareGrowthCurves" function of the statmod software package (11). For comparing treatment groups, a Mann–Whitney U test was used when n was <6 and a two-tailed Student t test when n was ≥6 and greater using GraphPad Prism 6 software.

**Results and Discussion**

The present study was prompted by the recent finding that stromal cells expressing the membrane protein, FAP, mediate immune suppression in immunogenic transplanted tumors established with the LL2/OVA cancer cell line (8). Because this report showed that the population of tumoral FAP+ cells comprised CD45+ and CD45− subsets, this first follow-up study addresses the important aim of de...
Thus, FAP is expressed by a minor proportion of tumoral macrophages that has an inflammatory M2 phenotype, being CCR2$^+$, MHCII$^lo$, CD11c$^lo$, and CD206$^+$ (Fig. 1C).

To determine whether this FAP$^+$ subset of tumoral F4/80$^{hi}$ macrophages had immune-suppressive function, we generated bone marrow chimeric mice by adoptively transferring male bone marrow from C57BL/6-Ly5.1 or FAP/DTR BAC Tg mice that had been depleted of FAP$^+$ cells into lethally irradiated female recipients that were either C57BL/6-Ly5.1, C57BL/6 (CD45.2), or FAP/DTR BAC Tg (Fig. 2A). In the resulting chimeric mice, peripheral blood leukocytes were always of at least 95% donor origin (Supplementary Fig. S2). The recipient

Figure 1. FAP expression by tumoral CD45$^+$ cells marks a subpopulation of F4/80$^{hi}$ macrophages. A, a representative FACS dot plot of gated CD45$^+$ cells from a single-cell suspension of an LL2/OVA tumor stained with the additional antibodies shown. B, the proportion of cells in five LL2/OVA tumors that were F4/80$^{hi}$ and either FAP$^+$ or FAP$^-$. C, FACS histograms of gated tumoral FAP$^+$ and FAP$^-$ F4/80$^{hi}$ cells stained with the specific antibodies shown (open) and isotype controls (shaded).

Figure 2. The selective depletion of DTR-expressing FAP$^+$CD45$^+$ and FAP$^-$/CD45$^+$ tumoral cells from bone marrow chimeric mice by the administration of DTX. A, a sketch depicting the donor-recipient combinations for generating the chimeric mice. B, the proportions of each of the two subsets of FAP$^+$ cells in LL2/OVA tumors in individual bone marrow chimeric mice that had received DTX for 3 consecutive days. C, the tumor volumes (left) and the calculated percent change in growth (right) of LL2/OVA tumors in the groups of bone marrow chimeric mice that were given DTX for 3 consecutive days. ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. 

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The origin of FAP^{+}/CD45^{-} cells in tumors was verified by a lack of the Y chromosome (data not shown). The bone marrow chimeric mice were challenged by subcutaneous injection of LL2/OVA cells. When tumors were established, mice were given DTX for 3 consecutive days, which led to the depletion of only the DTR-expressing subset of tumoral FAP^{+} cells (Fig. 2B). At the initiation of DTX there was no statistically significant difference in the tumor sizes between the non-Tg control and either DTR chimeric group (P > 0.05) (Supplementary Fig. S3). However, thereafter, there was slowing of tumor growth in both types of DTR chimeric mice, but not in mice lacking the expression of DTR in either FAP^{+} subset (Fig. 2C). Therefore, FAP^{+} cells of both hematopoietic and mesenchymal origin have immune-suppressive function, and, in this tumor model, both are required for tumor immune protection.

Having determined that the FAP^{+}/F4/80^{hi} stromal cells were capable of immune suppression in the LL2/OVA tumor, we sought a mechanism for this function. The findings of an association of the immunomodulatory enzyme, HO-1, with M2 macrophages (14), and of the M2 phenotype of the FAP^{+}/F4/80^{hi} tumoral cells (Fig. 1C) suggested the possibility that HO-1 may be involved in this process. Indeed, FAP^{+}/F4/80^{hi} cells could be found expressing HO-1 in frozen sections from the tumors grown in the bone marrow chimeric mice lacking the expression of DTR in either FAP^{+} subset (Supplementary Fig. S4). The FAP^{+}/F4/80^{hi} cells, although distributed throughout the tumor, tended to be localized toward the tumor periphery. To evaluate the relative contribution of FAP^{+} F4/80^{hi} cells to total tumoral HO-1, LL2 cells were implanted in FAP/EGFP BAC Tg mice in which FAP^{+} cells expressed HO-1 and EGFP, and frozen sections of the subsequent tumors were assessed by confocal microscopy for EGFP fluorescence, and expression of F4/80 and HO-1 (Fig. 3A). Across three tumors, 75 \pm 4.9% of the HO-1-expressing tumoral cells were double positive for EGFP and F4/80 (Fig. 3B). Moreover, HO-1 mRNA was chiefly detected in FACS sort-purified FAP^{+}/F4/80^{hi} cells (Fig. 3C). Therefore, the FAP^{+}/F4/80^{hi} stromal cell comprised the majority of HO-1-expressing cells in the LL2 tumor. It should be noted that there was comparatively little expression of the closely related enzyme HO-2 in LL2/OVA tumors compared with HO-1 (Supplementary Fig. S5).

To determine whether the expression of HO-1 by the tumoral FAP^{+}/F4/80^{hi} macrophages contributed to their immune suppressive function, C57BL/6 mice bearing established nonimmunogenic LL2 or immunogenic LL2/OVA tumors were treated with SnMP, a specific inhibitor of HO that has no identified off-target effects or toxicities. A response to a porphyrin-related inhibitor of HO-1, the most selective of which is SnMP, has been
considered to be indicative of the role of this enzyme in a biologic process (15–20). While inhibition of HO using SnMP did not affect the growth rate of the LL2 tumors (Fig. 4A), it significantly slowed the growth of immunogenic LL2/OVA tumors, with this response occurring as early as 24 hours after the initiation of treatment (Fig. 4A). The effect of SnMP on the growth of the LL2/OVA tumor was confirmed to be immune-mediated, as the inhibitor had no effect on the growth of LL2/OVA tumors in Rag2−/− mice (Fig. 4A).

A subcutaneous tumor established with a cell line derived from an autochthonous pancreatic ductal adenocarcinoma (PDA) also demonstrated the occurrence of FAP+/F4/80+/HO-1+ stromal cells (Supplementary Fig. S6A). Inhibition of HO-1 by the administration of SnMP to C57BL/6 mice bearing established PDA tumors, which induce an immune response to an unknown antigen(s), slowed tumor growth by a mechanism that was immune-mediated, as the inhibitor had no effect on the growth of LL2/OVA tumors in Rag2−/− mice (Fig. 4A).

The acute cessation of LL2/OVA growth induced by SnMP (Fig. 4B) was not associated with a change in the immune cell composition of the tumors (Supplementary Fig. S7A) or with an increase in the activation state of the tumoral CD8 T cells, as assessed by CD69 and Lamp-1 expression (Supplementary Fig. S7B). Immune control induced by SnMP did correlate with an increase in activated tumoral CD31+ tissue factor+ (TF, coagulation factor III, CD142) endothelial cells (Fig. 4B).

The T-cell cytokines, TNF-α and IFN-γ, act synergistically to activate endothelial cells (21). Because SnMP did not alter the mRNA levels of IFN-γ and TNF-α in the LL2/OVA tumors (Supplementary Fig. S8), inhibition of HO-1 may have sensitized endothelial cells to these cytokines. For example, one of the products of HO-1, carbon monoxide, suppresses the proapoptotic effects of TNF-α on endothelial cells (22). To address this possibility, mice bearing LL2/OVA tumors were treated with neutralizing antibodies to IFN-γ and TNF-α and then treated with SnMP. In the presence of these anticytokine antibodies, SnMP did not arrest the growth of LL2/OVA tumors (Fig. 4D), consistent with the activity of HO-1 in the tumor microenvironment being related to suppressing the response of endothelial cells to these cytokines.

Of the many studies of HO-1 and inflammation, perhaps the most relevant to the current findings are those demonstrating the ability of this enzyme and its product, carbon monoxide, to...
suppress rejection of allografts in rodent transplantation models (23). Although this earlier study did not determine which cells expressed HO-1, the present finding that HO-1 is predominantly expressed by tumoral FAP+/F4/80+ cells, when coupled with the demonstration of their immune-suppressive function, is consistent with the theme that the host response to cancers involves tissue-protective processes that occur in other biologic settings. While the molecular basis for the immune suppression that is mediated by the mesenchymal FAP+/CD45+ stromal cell remains to be determined, the present findings suggest that SnMP, which has been given to humans to inhibit heme oxygenases (19), may have clinical efficacy in overcoming tumoral immune suppression when HO-1–expressing stromal cells are found to be present.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: J.N. Arnold, M. Kraman, D.T. Fearon
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References

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