Modulation of T-Cell Activation by Malignant Melanoma Initiating Cells

Tobias Schatton, Ute Schütte, Natasha Y. Frank, Qian Zhan, André Hoerning, Susanne C. Robles, Jun Zhou, F. Stephen Hodi, Giulio C. Spagnoli, George F. Murphy, and Markus H. Frank

Abstract

Highly immunogenic cancers such as malignant melanoma are capable of inexorable tumor growth despite the presence of antitumor immunity. Thus, only a restricted minority of tumorigenic malignant cells may possess the phenotypic and functional characteristics needed to modulate tumor-directed immune activation. Here we provide evidence supporting this hypothesis. Tumorigenic ABCB5⁺ malignant melanoma initiating cells (MMICs) possessed the capacity to preferentially inhibit IL-2–dependent T-cell activation and to support, in a B7.2-dependent manner, induction of CD4⁺CD25⁺FoxP3⁺ regulatory T cells (Tregs). Compared with melanoma bulk cell populations, ABCB5⁺ MMICs displayed lower levels of MHC class I, aberrant positivity for MHC class II, and lower expression levels of the melanoma-associated antigens MART-1, ML-IAP, NY-ESO-1, and MAGE-A. Additionally, these tumorigenic ABCB5⁺ subpopulations preferentially expressed the costimulatory molecules B7.2 and PD-1, both in established melanoma xenografts and in clinical tumor specimens. In immune activation assays, MMICs inhibited mitogen-dependent human peripheral blood mononuclear cell (PBMC) proliferation and IL-2 production more efficiently than ABCB5⁻ melanoma cell populations. Moreover, coculture with ABCB5⁺ MMICs increased the abundance of Tregs, in a B7.2 signaling-dependent manner, along with IL-10 production by mitogen-activated PBMCs. Consistent with these findings, MMICs also preferentially inhibited IL-2 production and induced IL-10 secretion by cocultured patient-derived, syngeneic PBMCs. Our findings identify novel T-cell modulatory functions of ABCB5⁺ melanoma subpopulations and suggest specific roles for these MMICs in the evasion of antitumor immunity and in cancer immunotherapeutic resistance. Cancer Res; 70(2); 697–708.

©2010 AACR.

Introduction

Tumor initiation and growth resulting from the formation of tumor-initiating cells or cancer stem cells (CSC) is an intriguing concept that is being increasingly validated experimentally (1, 2). The CSC concept has important therapeutic implications because specific targeting of CSCs might represent a novel strategy to eradicate cancers currently resistant to systemic therapy (3–7). In human malignant melanoma, a highly therapy-resistant cancer (8), we recently identified a subpopulation enriched for malignant melanoma initiating cells (MMIC; ref. 6) based on the expression of the chemoresistance mediator ABCB5 (9–11). The frequency of ABCB5⁺ MMICs correlates with disease progression in human patients, and targeting of MMICs abrogates tumor growth in experimental tumor models (6). Consistent with these findings, induction of terminal differentiation in melanoma cells results in downregulation of ABCB5 (12). Furthermore, the ABCB5 gene is preferentially expressed by in vitro clonogenic melanoma cell subsets (13), by melanomas with enhanced tumorigenic capacity (14), and by melanoma cells derived from metastatic, as opposed to primary, tumor lesions (15).

Tumor initiation has been found to vary with the immune status of xenotransplantation recipients (6, 16–19). In human acute myeloid leukemia (AML), higher numbers of CD34⁺CD38⁻ cells were required to initiate leukemias in lesser immunocompromised (17) compared with more severely immunocompromised murine recipients (16). This suggests that some, but not all, of the CD34⁺CD38⁻ leukemia cells can be targets of host antitumor immunity, implying that there exists an immunoevasive subpopulation of leukemia-initiating cells. However, leukemia-initiating cells are not invariably contained within the CD34⁺CD38⁻ subset because CD34⁺CD38⁻ leukemia cells have also been found to exhibit leukemia-repopulating activity when immunologic effector mechanisms directed at CD38 sorting antibody–coated
AML cells are inhibited (19). Tumor initiation might also be influenced by host immune status in human melanoma, as indicated by a recent study that detected higher frequencies of cells capable of initiating melanoma xenografts when using more severely immunocompromised interleukin-2 receptor γ chain null (IL-2Rγ−/−) nonobese diabetic/severe combined immunodeficient (NOD/SCID) hosts (18) compared with findings in NOD/SCID recipients (6, 18). These observations and the higher rates of cancer development in immunocompromised patients (20) suggest a negative correlation between the degree of host immunocompetence and rates of tumor initiation and growth (21). Furthermore, they indicate that under conditions of relatively intact immunity, only a restricted minority of tumor cells (i.e., MMICs) might possess the phenotypic and functional characteristics to evade immune-mediated rejection in melanoma (21), an immunogenic cancer even in untreated human patients (22).

There are several mechanisms by which stem cells or MMICs might modulate immune responses (8, 21, 23, 24), including induction of T-cell anergy, generation of regulatory T (Treg) cells, secretion of immunosuppressive cytokines, or downregulation of melanoma-associated antigen (MAA; refs. 20, 21). According to the “two-signal” paradigm, antigen-dependent T-cell activation requires two distinct signals: signal 1 is provided through T-cell receptor engagement with the MHC/antigenic peptide complex, and signal 2 through costimulatory pathways, leading to either full activation through positive costimulatory signals or impaired T-cell activation through so-called negative costimulatory signals (25). These signals may also be involved in tumor evasion of host immunity (20).

We hypothesized that ABCB5+ melanoma subpopulations, enriched for MMICs (6), differ from melanoma bulk populations with respect to the expression of clinically relevant immunodeterminants, and that ABCB5+ cells, based on a unique immunophenotype, possess the functional characteristics to preferentially inhibit human lymphocyte responses required for antitumor immunity.

Materials and Methods

**Tumor cell isolation, flow cytometry, and real-time quantitative reverse transcription-PCR.** Clinical melanoma cells were derived from surgical specimens according to Institutional Review Board (IRB)-approved research protocols. Single-cell suspensions were generated using collagenase as described (6). ABCB5+/-, B7.2+/-, PD-1+/- subpopulations were generated using anti-ABCB5, anti-B7.2, or anti-PD-1 monoclonal antibody (mAb) labeling, respectively, followed by magnetic bead cell sorting as described (6, 9). Coexpression of ABCB5 with signal 1- and signal 2-associated molecules and MAAs on patient-derived or established melanoma cells was determined by flow cytometry as described previously (6, 9). Levels of mRNA expression of transforming growth factor-β (TGF-β) pathway molecules were assayed and statistically analyzed by real-time quantitative reverse transcription-PCR (RT-PCR) as described (9, 26), using a human TGF-β/bone morphogenetic protein signaling PCR Array (PAHS-035, SA Biosciences) according to the manufacturer’s instructions.

**Antibodies.** The specific IgG1: anti-ABCB5 mAb 3C2-D12 (6, 9) was used in the herein reported studies. Allophycocyanin (APC)-labeled anti-ABCB5 mAb was custom manufactured by Invitrogen by conjugating fluorescent APC organic dye to the 3C2-D12 mAb. Unconjugated or phycoerythrin (PE)-conjugated IgG1, IgG2a, IgG2b, and IgG3: FITC-conjugated IgG1 mouse isotype control mAbs; unconjugated mouse anti-human B7.2 and PD-1 mAbs; PE-conjugated mouse anti-human 4-1BB, B7.1, B7.2, CD4, CD28, CD31, CD40, CD45, CD70, CTLA-4, HLA-ABC, HLA-DR, ICOS, PD-1, PD-L1, and PD-L2 mAbs; FITC-conjugated anti-human CD25 mAb and goat anti-mouse IgG secondary antibody; as well as unconjugated and biotin-conjugated anti-human IL-2, IL-4, IL-5, and IL-10 mAbs were purchased from BD Biosciences. Unconjugated and biotin-conjugated anti-human IFN-γ mAbs were purchased from Thermo Fisher Scientific. PE-conjugated mouse anti-human 4-1BB, CD40L, ICOSL, and OX40L mAbs were purchased from BioLegend. PE-conjugated anti-human CD27 mAb was purchased from Immunotech. APC-conjugated secondary and neutralizing antihuman B7.2 and IgG2b isotype control mAbs were purchased from eBioscience. APC-conjugated IgG1 mouse isotype control and antihuman FoxP3 mAbs were purchased from Miltenyi Biotec. Unconjugated mouse anti-human MART-1 mAb was purchased from Abcam and mouse anti-human ML-IAP from Imgenex. The specific mouse anti-human D8.38 and 57B mAbs, used for the herein reported flow cytometric detection of NY-ESO-1 and MAGE-A, respectively, were generated as described previously (27, 28). For immunofluorescence staining, unconjugated goat anti-human PD-1 and B7.2 antibodies were purchased from R&D Systems and rabbit anti-human PD-L1 antibody was from Lifespan Biosciences. Secondary Alexa Fluor (AF) 488–conjugated donkey anti-mouse IgG, AF 594–conjugated donkey anti-rabbit IgG, and AF 594–conjugated donkey anti-goat IgG antibodies were purchased from Invitrogen. Goat IgG (Jackson Immunoresearch) and rabbit IgG (Bethyl Laboratories) antibodies were used as negative controls.

**Human melanoma xenotransplantation and immunohistochemistry.** NOD/SCID mice were maintained under defined conditions in accordance with institutional guidelines and experiments were done according to approved experimental protocols. For tumorigenicity studies, B7.2+/-, PD-1+/- melanoma cells were injected s.c. into recipient NOD/SCID mice and tumor formation/growth was determined up to 8 wk, as described (6). For immunohistochemical analysis, unsegregated melanoma cells were injected s.c. into flanks of recipient NOD/SCID mice. Resultant tumor xenografts or patient-derived melanoma biopsy specimens were stained for coexpression of ABCB5 with the B7.2, PD-1, or PD-L1 markers by immunofluorescence double labeling, as described (6).

**Peripheral blood mononuclear cell proliferation, enzyme-linked immunosorbent spot, and ELISA assays.** Human peripheral blood mononuclear cells (PBMC) were isolated from whole blood samples by Ficoll-Paque density
gradient centrifugation as described (29), according to IRB-approved protocols of the Dana-Farber Cancer Institute and Children’s Hospital Boston. To determine the effect of melanoma cells on PBMC proliferation, irradiated (7,000 rad) unsegregated, ABCB5+, or ABCB5− tumor populations were cocultured with freshly isolated PBMCs (1:10 ratio) in the presence of phytohemagglutinin (PHA). PBMC proliferation was assessed by quantification of [3H]thymidine incorporation as described (29). For determination of cytokine production (IFN-γ, IL-2, IL-4, IL-5, or IL-10) in experimental groups as above, enzyme-linked immunosorbent spot (ELISPOT) analyses were done as described previously (29). For determination of cell death, Annexin V-PE/7-amino-actinomycin D (7-AAD) staining followed by flow cytometric

Figure 1. Identification of signal 1 and signal 2 members of immune activation on melanoma cells. A, representative flow cytometric analyses of melanoma cells stained for MHC class I or MHC class II antigens. Single-color flow cytometry analyses of melanoma specimens for expression of costimulatory molecules (B and C) and MAAs (D). Horizontal bars, mean. Bottom rows, representative histogram plots showing marker-stained populations (red) compared with isotype-stained controls (shaded).
Figure 2. Immunophenotype of ABCB5+ MMICs. Expression of MHC class I or MHC class II molecules (A) and of costimulatory molecules (B and C) by ABCB5+ versus ABCB5− malignant melanoma cells (MMC) as determined by dual-color flow cytometry. Columns, mean; bars, SEM (*, P < 0.05). Bottom rows, representative histogram plots showing marker-stained populations (red) compared with isotype-stained controls (shaded). D, immunofluorescence double staining of clinical melanoma sections (left) and melanoma xenograft sections (right) for coexpression of ABCB5 (AF 488, green) with B7.2 (AF 594, red), PD-1 (AF 594, red), or PD-L1 (AF 594, red). Nuclei are visualized by staining with 4′,6-diamidino-2-phenylindole (blue).
analysis was done in experimental groups as above, as described previously (30). Treg cell frequencies were determined by measuring the proportion of CD4\(^+\)CD25\(^+\)FoxP3\(^+\) T cells using triple-color flow cytometry, as described previously (6). To examine the immunomodulatory capacity of ABCB5\(^-\) vis-à-vis ABCB5\(^+\) melanoma cells in coculture with syngeneic PBMCs, irradiated ABCB5-sorted patient-derived melanoma cells were cocultured with donor-identical PBMCs (1:1 ratio) in the absence of mitogenic stimulation, with determination of IL-2 and IL-10 production in coculture supernatants by ELISA as described previously (29).

Results

Identification of signal 1 and signal 2 members of immune activation on human melanoma cells. We first characterized MHC antigen expression levels (signal 1) in clinical and established melanoma cells because of the central relevance of MHC molecules in the immune recognition of transformed cells and because of the established association of a continuous loss of class I and an increase in class II MHC expression with melanoma progression, metastatic spread, and more aggressive tumor growth (31–34). Single-color flow cytometry analysis revealed MHC class I to be consistently expressed by the majority of melanoma cells (98.0 ± 0.2%, mean ± SEM, n = 4; Fig. 1A; Supplementary Table S1). In contrast, MHC class II was only expressed on a minority population of melanoma cells ranging from 0.2% to 1.4% of cells (Fig. 1A; Supplementary Table S1). These results indicated that melanoma cells possess the capacity to deliver signal 1 of T-cell activation. We next examined systematically the expression of signal 2-associated costimulatory molecules to determine whether melanoma cells might possess the capacity to modulate immune responses through positive or so-called negative, often T-cell activation–impairing, costimulatory signals. Immunophenotypic characterization with respect to members of the tumor necrosis factor (TNF): TNF receptor (TNF-R) superfamily of costimulatory molecules (expression levels summarized in Supplementary Table S1) revealed expression of CD40 by only 1.8 ± 0.6% and of CD40L by 2.3 ± 2.1% of G3361 melanoma cells (mean ± SEM, n = 5 repeat experiments), and A375 (n = 5) or patient-derived melanoma cells (n = 2) did not exhibit any positivity for either marker (Fig. 1B). Furthermore, 4-1BB, CD27, or CD70 were also not expressed by G3361, A375, or patient-derived melanoma cells (median percentage 0.0%, n = 10; Fig. 1B). In contrast, 4-1BBL (11.1 ± 2.0%, n = 10), OX40 (7.5 ± 2.2%, n = 11), and OX40L (96.0 ± 1.9%, n = 6) were consistently and significantly expressed by G3361, A375, or patient-derived melanoma cells (Fig. 1C). Further immunophenotypic characterization directed at members of the CD28:B7 superfamily of costimulatory molecules (expression levels summarized in Supplementary Table S1) showed no significant expression levels of the costimulators CD28, B7.1, or ICOS, or of the costimulatory ligand PD-L2 (median percentage 0.0%, n = 10–14; Fig. 1C). However, melanoma cells expressed significant amounts of the costimulatory receptor CTLA-4 (4.0 ± 1.6%, mean ± SEM, n = 6) and its ligand B7.2 (1.8 ± 0.5%, n = 13), as well as of the costimulatory molecule ICOSL (2.1 ± 0.4%, n = 13; Fig. 1C). In addition, the costimulatory receptor PD-1 (3.0 ± 0.7%, n = 15) and its ligand PD-L1 (3.9 ± 0.7%, n = 14) were significantly expressed by both established and patient-derived melanomas (Fig. 1C). With regard to MAA, flow cytometry analysis revealed MART-1 and MLAGP (35) to be expressed by 2.5 ± 11.1% (mean ± SEM, n = 10) and 26.1 ± 11.4% (n = 9) of melanoma cells, respectively, and the cancer testis antigens NY-ESO-1 (28) and MAGE-A (27) by 24.5 ± 10.0% (n = 11) and 49.0 ± 7.9% (n = 12) of melanoma cells, respectively (Fig. 1D; Supplementary Table S1). Thus, human melanoma cells express both signal 1 and signal 2 members of immune activation as well as MAAs, which suggested a capacity to functionally modulate host immunity.

ABCB5\(^-\) MMIC–enriched melanoma subsets, but not ABCB5\(^+\) melanoma bulk populations, possess signal 1, signal 2, and MAA immunophenotype associated with immune-evasive capacity. Because of the established association between patterns of MHC molecule expression with immune evasion, tumorigenicity, and disease progression (31–34), and based on our results that both MHC class I and class II antigens were heterogeneously expressed on human melanoma cells (Fig. 1A), we next determined the distribution of these molecules on ABCB5\(^-\) subsets vis-à-vis ABCB5\(^+\) melanoma bulk populations. This was assessed by immunofluorescence double staining for ABCB5 and MHC class I or class II and subsequent dual-color flow cytometric analysis. We found that MHC class I expression was significantly reduced on ABCB5\(^-\) compared with ABCB5\(^+\) melanoma cells (73.3 ± 7.0% versus 98.0 ± 0.2%, respectively, mean ± SEM, n = 4, P < 0.05; Fig. 2A). Furthermore, we detected ABCB5\(^+\) melanoma cells that completely lacked MHC class I expression (Fig. 2A). In contrast, MHC class II molecules were expressed selectively on ABCB5\(^-\) cells but not on ABCB5\(^+\) bulk populations (4.8 ± 1.9% versus 0.5 ± 0.3%, respectively, not significant [NS], n = 4, P > 0.05; Fig. 2A; results summarized in Supplementary Table S1).

Further characterization with respect to costimulatory molecules of the TNF/TNF-R superfamily (results summarized in Supplementary Table S1) revealed preferential expression on ABCB5\(^-\) versus ABCB5\(^+\) melanoma cells of 4-1BBL (43.3 ± 9.1% versus 8.1 ± 1.7%, respectively, n = 10, P < 0.05) and reduced expression of OX40L (82.0 ± 6.7% versus 95.6 ± 2.6%, respectively, n = 6, P < 0.05; Fig. 2B). In contrast, the respective costimulatory receptors 4-1BB or OX40 were expressed at similar levels on ABCB5\(^-\) and ABCB5\(^+\) melanoma subsets (1.4 ± 1.0% versus 0.1 ± 0.2%, n = 10, NS, and 18.7 ± 5.8% versus 13.2 ± 7.6%, n = 11, NS, respectively; Fig. 2B). Further immunophenotypic characterization with regard to costimulatory molecules of the CD28:B7 superfamily (results summarized in Supplementary Table S1) revealed B7.2 to be significantly overexpressed by ABCB5\(^-\) versus ABCB5\(^+\) subpopulations (12.0 ± 3.4% versus 1.3 ± 0.3%, n = 10, respectively, P < 0.01), whereas CD28 (3.7 ± 2.1% versus 0.1 ± 0.0%, n = 10, NS), CTLA-4 (6.6 ± 1.7% versus 5.0 ± 2.3%, n = 6, NS), and B7.1 (0.3 ± 0.2% versus 0.1 ± 0.0%, n = 10, NS) were not differentially expressed by ABCB5\(^-\) versus ABCB5\(^+\) melanoma populations (Fig. 2C).
Reduced expression by ABCB5+ versus ABCB5− melanoma cells was shown for the costimulator ICOSL (0.5 ± 0.2% versus 1.9 ± 0.4%, n = 12, P < 0.01) but not for its receptor ICOS (0.1 ± 0.1% versus 0.0 ± 0.0%, n = 12, NS), which was not found to be expressed at significant levels in either melanoma subset (Fig. 2C). Among members of the programmed death family of negative costimulators, PD-1 was preferentially expressed by ABCB5+ compared with ABCB5− melanoma cells (10.5 ± 2.4% versus 2.5 ± 0.7%, n = 12, P < 0.05; Fig. 2C). In contrast, the PD-1 ligand PD-L1 was expressed at significantly lower levels on ABCB5+ compared with ABCB5− tumor cells (1.1 ± 0.4% versus 3.1 ± 0.7%, n = 13, P < 0.05; Fig. 2C). In contrast, also consistent with flow cytometry analyses, regions of positivity for PD-L1 were cytologically distinct from ABCB5+ tumor subsets (Fig. 2D). We further confirmed selective coexpression of B7.2 and PD-1 with ABCB5 on human melanoma cells using human-specific antibodies in melanoma xenografts to murine hosts (Fig. 2D).

To confirm that B7.2+ and PD-1+ melanoma cells that preferentially coexpressed ABCB5 were enriched for MMICs, we compared the abilities of B7.2+ versus B7.2− and of PD-1+ versus PD-1− melanoma cells to initiate tumor formation in vivo, using primary patient-derived tumor cells in human to NOD/SCID mouse xenotransplantation experiments. B7.2- and
PD-1–dependent cell sorting was done using immunomagnetic selection, followed by confirmation of purity and viability of sorted populations, as described (6). This isolation technique resulted in purities of sorted melanoma cells >95%, as opposed to CD45+ or CD31+ stromal cells, similar to results described previously (6). Groups of mice were injected s.c. with replicate (n = 10) inocula of B7.2+ or B7.2− and of PD-1+ or PD-1− melanoma cells representing three distinct patients over a log-fold range from cell doses unable to efficiently initiate tumor growth (10^4 cells) to doses that consistently initiated tumor formation when ABCB5+ cells were used (6). Consistent with our findings of a preferential expression of B7.2 and PD-1 on ABCB5+ melanoma cells, we found that purified B7.2+ tumor subsets expressed significantly more ABCB5 than did B7.2− bulk populations (86.7 ± 3.7% versus 6.3 ± 2.6%, respectively, mean ± SEM, n = 3, P < 0.01) and PD-1+ fractions more ABCB5 than did their PD-1− counterparts (70.7 ± 4.1% versus 10.1 ± 1.5%, respectively, n = 3, P < 0.01). Of 30 aggregate mice injected with B7.2− melanoma cells, only 5 transplanted with the highest cell dose generated a tumor. In contrast, 19 of 30 mice injected with B7.2+ populations formed tumors (P < 0.01; Fig. 3A; Supplementary Table S2), showing that B7.2 is preferentially expressed on tumorigenic ABCB5+ melanoma cells (enrichment of tumorigenicity: 21-fold, P < 0.01; Fig. 3A). Similarly, PD-1+ melanoma subsets preferentially formed tumors compared with PD-1− melanoma bulk populations in 16 of 30 compared with 4 of 30 recipient mice (P < 0.01), respectively (Fig. 3B; Supplementary Table S3), identifying PD-1 to be also expressed on ABCB5+ cells with increased tumorigenic capacity (enrichment of tumorigenicity: 19-fold, P < 0.01; Fig. 3B).

We next examined whether MAAs are differentially expressed by ABCB5+ melanoma cells vis-à-vis ABCB5− melanoma bulk populations (results summarized in Supplementary Table S1). We found that ABCB5+ melanoma subpopulations consistently expressed lower levels of the MAAs ML-IAP, NY-ESO-1, and MAGE-A compared with ABCB5− melanoma bulk populations in all clinical patient-derived and established melanoma cells examined (ML-IAP: 0.8 ± 0.6% versus 11.4 ± 4.4%, n = 8; NY-ESO-1: 0.3 ± 0.1% versus 11.4 ± 7.1%, n = 9; MAGE-A: 2.3 ± 1.2% versus 47.1 ± 11.1%, n = 8, P < 0.01, respectively; Fig. 4A and C). ABCB5+ melanoma cells also expressed significantly lower levels of MART-1 compared with ABCB5− tumor populations [18.3 ± 13.4% versus 20.9 ± 13.9%, n = 6, P < 0.05 (Wilcoxon matched pairs test); Fig. 4C]. However, whereas MART-1 was expressed at lower levels in ABCB5+ melanoma subpopulations across all clinical patient samples examined (Fig. 4B), consistent with its expression pattern in established A375 melanoma cells described previously (21), the molecule was not found differentially expressed in ABCB5+ versus ABCB5− subsets in established G3361 melanoma cells (Fig. 4B), indicating a greater variability of this marker, compared with other MAAs, with regard to expression in ABCB5+ melanoma cells.

With regard to TGF-β signaling pathway members, previously found to be expressed in human melanoma cells and
implicated in immunomodulation (20, 21), ABCB5+ melanoma cells, in comparison with ABCB5− melanoma populations, expressed higher mRNA levels of TGFB2 (fold change: 2.3 ± 0.7, mean ± SEM, $P < 0.05$), TGFB3 (2.6 ± 0.8, $P < 0.05$), TGFB1 (2.0 ± 0.7, $P < 0.05$), and TGFBR1 (3.0 ± 0.7, $P < 0.05$), as determined by quantitative RT-PCR (Fig. 4D). No significant differences were observed for ABCB5+ vis-à-vis ABCB5− melanoma cells for TGFB1, TGFB1I1, TGFBR2, TGFBR3, TGFBRAP1, or TGIF1 mRNA expression levels (Fig. 4D).

In summary, ABCB5+ melanoma cells, unlike cancer bulk populations, express low levels of MHC class I and are positive for MHC class II, a signal 1 phenotype associated with poor clinical outcome (31–34). Moreover, this melanoma subpopulation selectively expresses the costimulatory molecules B7.2 and PD-1, which represent targets in clinical melanoma immunotherapy (36), and expresses reduced levels of immunogenic MAAs. Hence, we next examined the effect of ABCB5+ MMIC–enriched subsets on immune activation.

**ABCB5+ MMIC–enriched melanoma subsets preferentially inhibit T-cell activation.** To dissect functionally a potential role of ABCB5+ melanoma subpopulations in the inhibition of T-cell activation, we examined the effects of either unsegregated or ABCB5-sorted melanoma populations on mitogen-induced human lymphocyte proliferation and cytokine secretion and on cytokine secretion in cocultures with syngeneic PBMCs. Addition of unsegregated human malignant melanoma cells to mitogen-stimulated PBMC cultures resulted in a significant inhibition of 83.8% of human lymphocyte proliferation compared with PHA-stimulated PBMC controls ($P < 0.0001$; Fig. 5A). Consistent with the
observed suppression of proliferation, we found that melanoma cells inhibited secretion of IL-2, a key stimulator of cytotoxic T-cell growth, activation, and differentiation (38), by 87.2% \((P < 0.01; \text{Fig. 5}A)\). With regard to additional cytokines, coculture of mitogen-activated PBMCs with human melanoma cells increased production of the human cytokine synthesis inhibitory factor, IL-10 (2.3-fold, \(P < 0.01; \text{Fig. 5}B\)), and the hallmark cytokine of T helper (Th) 1 cells (25), IFN-γ (4.5-fold, \(P < 0.01; \text{Fig. 5}C\)). Furthermore, coculture inhibited production of the Th2 cytokine and key mediator of B-cell growth (25), IL-5, by 70.9% \((P < 0.01; \text{Fig. 5}D)\), and had no effect on induction of IL-4 (NS; Fig. 5D), a cytokine induced during differentiation of naïve Th cells into Th2 (25).

To investigate whether these melanoma-induced effects were preferentially driven by ABCB5+ MMIC–enriched sub-sets, we first compared the ability of ABCB5+ versus ABCB5− tumor cells to inhibit mitogen-activated PBMC proliferation. ABCB5+ subpopulations blocked mitogen-stimulated PBMC proliferation by 93.0% \((P < 0.0001; \text{Fig. 6}A)\). This inhibitory effect was significantly greater than that exerted by ABCB5− bulk populations (10,660 ± 1,406 versus 22,170 ± 1,343 cpm, mean ± SD, respectively, \(P < 0.0001; \text{Fig. 6}A\)). To exclude apoptotic cell death as a potential cause of inhibited proliferation, we quantified cell death by Annexin V-PE/7-AAD staining and flow cytometry. We found that neither ABCB5+ nor ABCB5− melanoma cells induced cell death in

---

**Figure 6.** ABCB5+ MMICs preferentially inhibit T-cell activation. A, \([3H]\) thymidine uptake (left) and cell death (Annexin V-PE/7-AAD staining (right)) of human PBMCs cultured with or without PHA in the presence or absence of ABCB5+ or ABCB5− malignant melanoma cells. Columns, mean (representative of \(n = 3–6\) independent experiments); bars, SD. *, \(P < 0.05\). B, fold differences of cytokine secretion by mitogen-stimulated PBMCs cultured in the presence of ABCB5+ versus ABCB5− melanoma cells as determined by ELISPOT analysis. Columns, mean (representative of \(n = 4\) independent experiments); bars, SEM. Bottom, images of representative ELISPOT wells. C, effects of B7.2 blockade in ABCB5+ or ABCB5− melanoma cells on cocultured mitogen-stimulated PBMCs with regard to IL-10 production (spots per well; left) and Treg cell frequencies (percent CD4+CD25+FoxP3+ triple-positive PBMCs; right). Columns, mean (representative of \(n = 3–5\) independent experiments); bars, SEM. D, IL-2 (left) and IL-10 (right) production by PBMCs cultured in the presence of patient-derived, syngeneic ABCB5+ versus ABCB5− melanoma subpopulations, as determined by ELISA. Columns, mean cytokine concentrations (pg/mL) for \(n = 3\) replicate wells of \(n = 6\) independent experiments; bars, SEM.
mitogen-activated PBMCs above baseline levels (percent cell death: 22.1 ± 4.1% versus 22.5 ± 6.3% versus 18.0 ± 1.1%, mean ± SD, NS, respectively; Fig. 6A). As a correlate of enhanced blockage of proliferation, ABCB5+ melanoma cells inhibited IL-2 production by mitogen-stimulated PBMCs by 75.8% (P < 0.0001), an effect also significantly greater than that of ABCB5− populations (P < 0.05; Fig. 6B). Moreover, ABCB5+, but not ABCB5−, subsets inhibited IL-4 production by 18.5 ± 4.5% (mean ± SEM, P < 0.01). IL-5 production was also preferentially inhibited by ABCB5+ tumor subpopulations compared with ABCB5− melanoma cells, by 25.2 ± 4.5% (mean ± SEM, P < 0.001). In contrast, both ABCB5+ and ABCB5− melanoma cells increased PBMC secretion of IL-10 and IFN-γ at similar rates (Fig. 6B). However, only in the case of coculture with ABCB5+ melanoma cells was the induction of IL-10 dependent on signaling through B7.2 because blockade of B7.2 on ABCB5+ subsets, but not on B7.2-negative ABCB5+ melanoma bulk populations, resulted in significant inhibition of IL-10 secretion by 24.2% compared with controls (P < 0.05; Fig. 6C). Consistent with these findings, blockade of B7.2 on ABCB5+ melanoma cells during coculture with mitogen-activated PBMCs selectively inhibited induction of CD4+CD25+FoxP3+ Treg cells, a major cellular source of IL-10 (39), by 34.8% compared with controls (P < 0.05; Fig. 6C).

To confirm the preferential immunomodulatory capacity of ABCB5+ melanoma subsets vis-à-vis ABCB5− melanoma bulk populations also in an in vitro assay closely resembling the melanoma patient environment, we cocultured ABCB5−sorted patient-derived melanoma subpopulations with syngeneic, donor-identical PBMCs in the absence of mitogenic stimulation. Consistent with the preferential inhibition of IL-2 secretion of cocultured mitogen-stimulated PBMCs by ABCB5+ tumor subpopulations, we found that ABCB5+ melanoma subsets did not induce IL-2 release by cocultured syngeneic PBMCs (0.0 ± 0.0 pg/mL, mean ± SEM, n = 3; Fig. 6D). In contrast, significant levels of IL-2 (4.6 ± 0.5 pg/mL, n = 3, P < 0.01) were detected in supernatants of PBMCs cocultured with ABCB5+ melanoma cells (Fig. 6D). The lack of induction of IL-2 production by ABCB5+ MIC indicates that ABCB5+ melanoma cells promote IL-2–driven T-cell–mediated antitumor immune responses, which include IL-2–dependent cytotoxic T-cell function (38), significantly less than do ABCB5− melanoma bulk populations. Furthermore, we found that ABCB5+ tumor subpopulations induce secretion of the immunosuppressive cytokine IL-10 by cocultured syngeneic PBMCs 3.9-fold more efficiently than do ABCB5− bulk populations (19.3 ± 2.3 versus 5.0 ± 1.9 pg/mL, n = 3, respectively, P < 0.01; Fig. 6D). Our findings that ABCB5+ MIC–enriched melanoma subsets block PBMC proliferation and IL-2 production more efficiently than do ABCB5− cells and preferentially induce IL-10 production in syngeneic coculture identify a novel role of MICs in the modulation of T-cell activation.

Discussion

Our results establish immunomodulatory functions of ABCB5+ melanoma subpopulations enriched for MICs and identify mechanisms, including IL-2 inhibition and B7.2-dependent IL-10 induction, through which these tumorigenic cancer subpopulations inhibit antitumor immunity. These functions might account for specific growth advantages of MICs in a developing tumor in relatively immunocompetent hosts, as has previously been postulated (6, 18). Furthermore, identification of modulation of T-cell activation by MICs has important implications for current immunotherapeutic modalities in human malignant melanoma as well as for the design of novel therapeutic strategies that not only target bulk populations of tumor cells but also reverse ABCB5+ MIC–mediated immunomodulation.

Our study identifies inhibition of IL-2 as one mechanism through which ABCB5+ melanoma cells evade host antitumor immunity and potentially also escape immunotherapy. Previous studies have suggested an inverse correlation of IL-2/IL-2R signaling with tumorigenic growth (40, 41). Moreover, tumorigenic melanoma cell frequencies are significantly enhanced in IL-2Rγ−/− compared with IL-2RγWT murine NOD/SCID recipients (6, 18). Preferential inhibition of IL-2 production by ABCB5+ melanoma cells provides a possible explanation for these observed differences (6, 18). Specifically, tumorigenicity experiments conducted in the absence of IL-2 signaling (18) might overestimate MIC frequencies (1, 21) because tumor host environments characterized by absent antitumor immunity might permit tumor bulk populations, which do not normally initiate tumors and might not possess MIC-specific self-renewal and differentiation capacity, to also cause experimental tumor growth (21).

In addition to IL-2 inhibition, our study defines further immunophenotypic differences and immunomodulatory functions of ABCB5+ versus ABCB5− melanoma cells. First, we identify lower or absent MHC class I expression and aberrant positivity for class II to be characteristic of ABCB5+ melanoma cells. This phenotype, like ABCB5 positivity (6), is associated with clinical melanoma progression (31–34) and, furthermore, with therapeutic unresponsiveness and adverse clinical outcome in human patients (31–34). Because MHC class I downregulation represents one of the foremost mechanisms used by tumor cells to evade host antitumor immunity (31), reduced MHC class I expression by ABCB5+ melanoma cells suggests relative immune privilege and resistance to immune-mediated rejection. Second, ABCB5+ MIC–enriched subsets express markedly reduced levels of the MAAs MART-1, ML-IAP, MAGE-A, and NY-ESO-1 compared with ABCB5− bulk populations, also indicating enhanced immunoevasive properties of tumorigenic MICs. A previous study detected NY-ESO-1 expression on CD133+ melanoma subpopulations (42). However, this subset has not been established as a MIC subset (2), nor does CD133 positivity coincide fully with or serve as a surrogate marker for ABCB5+ melanoma subpopulations (9). Importantly, our finding that patient–derived ABCB5+ tumor subsets are relatively negative for MAAs suggests that treatment strategies using activated, MAA-reactive CTLs (22, 43) might fail to consistently target all tumorigenic MICs. Third, the preferential expression of B7.2 and PD-1 could confer additional immunoevasive and protumorigenic properties on ABCB5+ subpopulations because T-cell anergy...
can result from distinct costimulatory signals on T-cell receptor engagement (25). For example, B7.2 signaling can promote T-cell activation and differentiation, including induction of Treg cells required for immunologic tolerance (44). Specifically, our study shows that B7.2 expressed by ABCB5+ tumor subsets, similar to its role in other systems (45), functions in CD4+CD25+FoxP3+ Treg cell induction and regulates secretion of the Treg cell product IL-10 (39). Of note, further potential mechanisms of ABCB5+ melanoma cell–induced immunomodulation might exist, as is indicated by our finding that these subpopulations also express increased transcript levels of a subset of TGF-β pathway members compared with ABCB5− melanoma cells, including TGFβ2 and TGFβ3, both of which have been previously implicated in Treg cell activation (46, 47), providing the rationale for further studies about the potential roles of TGF-β signaling by ABCB5+ tumor cells in the context of antitumor immunity.

The recognition of MMIC-associated immunomodulation is highly relevant to human melanoma therapy, as it provides the rationale to examine in clinical trials whether current approved or investigational immunotherapeutic strategies that use IL-2 (48) or target the B7-CD28/CTLA-4 or PD-1 signaling pathways in melanoma patients (37, 49) might function, in part, to inhibit ABCB5+ MMIC–induced tumor immune evasion and immunologic tolerance. Although further investigations are needed to establish whether tumor-initiating cell–associated immunomodulation also occurs in other solid tumors, our results represent a significant first step in dissecting the relationship between MMICs and antitumor immunity in human malignant melanoma.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Dr. Meenhard Herlyn (Wistar Institute, Philadelphia, PA) and Dr. Waaga-Gasser (University of Würzburg, Würzburg, Germany) for providing melanoma specimens.

Grant Support

NIH/National Cancer Institute grants 1RO1CA113796-01AI and 1RO1CA138231-01 (M. H. Frank) and grant 2P50CA093683-06A20006 (M. H. Frank and G. F. Murphy). T. Schatton is the recipient of a Postdoctoral Fellowship Award from the American Heart Association Founders Affiliate.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 5/5/09; revised 10/26/09; accepted 11/12/09; published OnlineFirst 1/12/10.

References

Modulation of T-Cell Activation by Malignant Melanoma Initiating Cells

Tobias Schatton, Ute Schütte, Natasha Y. Frank, et al.

Cancer Res 2010;70:697-708. Published OnlineFirst January 12, 2010.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-09-1592

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2010/01/11/0008-5472.CAN-09-1592.DC1

Cited articles
This article cites 49 articles, 18 of which you can access for free at:
http://cancerres.aacrjournals.org/content/70/2/697.full.html#ref-list-1

Citing articles
This article has been cited by 18 HighWire-hosted articles. Access the articles at:
/content/70/2/697.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.