ONCOIMMUNOLOGY
2016, VOL. 5, NO. 12, e1240857 (14 pages)
http://dx.doi.org/10.1080/2162402X.2016.1240857

Taylor & Francis
Taylor & Francis Group

ORIGINAL RESEARCH

OPEN ACCESS

Human melanomas and ovarian cancers overexpressing mechanical barrier molecule genes lack immune signatures and have increased patient mortality risk

Elise P. Salerno[a], Davide Bedognetti[b,c], Ileana S. Mauldin[a], Donna H. Deacon[a], Sofia M. Shea[a,d], Joel Pinczewski[a], Joseph M. Obeid[a], George Coukos[e], Ena Wang[c], Thomas F. Gajewski[f], Francesco M. Marincola[c], and Craig L. Slingluff Jr.[a]

[a]Division of Surgical Oncology, Department of Surgery, University of Virginia, Charlottesville, VA, USA; [b]Infectious Disease and Immunogenetics Section (IDIS), Department of Transfusion Medicine, Clinical Center and Trans-NIH Center for Human Immunology (CHI), National Institutes of Health, Bethesda, MD, USA; [c]Sidra Medical and Research Center, Doha, Qatar; [d]Department of Pathology, University of Virginia Health System, Charlottesville, VA, USA; [e]Ludwig Institute for Cancer Research, University of Lausanne, Lausanne, Switzerland; [f]Department of Medicine, University of Chicago, Chicago, IL, USA

ABSTRACT

We have identified eight genes whose expression in human melanoma metastases and ovarian cancers is associated with a lack of Th1 immune signatures. They encode molecules with mechanical barrier function in the skin and other normal tissues and include filaggrin (FLG), tumor-associated calcium signal transducer 2 (TACSTD2), and six desmosomal proteins (DST, DSC3, DSP, PPL, PKP3, and JUP). This association has been validated in an independent series of 114 melanoma metastases. In these, DST expression alone is sufficient to identify melanomas without immune signatures, while FLG and the other six putative barrier molecules are overexpressed in a different subset of melanomas lacking immune signatures. Similar associations have been identified in a set of 186 ovarian cancers. RNA-seq data from 471 melanomas and 307 ovarian cancers in the TCGA database further support these findings and also reveal that overexpression of barrier molecules is strongly associated with early patient mortality for melanoma ($p = 0.0002$) and for ovarian cancer ($p < 0.01$). Interestingly, this association persists for FLG for melanoma ($p = 0.012$) and ovarian cancer ($p = 0.006$), whereas DST overexpression is negatively associated with $CD8^+$ gene expression, but not with patient survival. Thus, overexpression of FLG or DST identifies two distinct patient populations with low immune cell infiltration in these cancers, but with different prognostic implications for each. These data raise the possibility that molecules with mechanical barrier function in skin and other tissues may be used by cancer cells to protect them from immune cell infiltration and immune-mediated destruction.

Abbreviations: APC2, adenomatosis polyposis coli 2; BCAT, β-catenin; BPAG1, bullous pemphigoid antigen 1; CCR5, C–C chemokine receptor type 5, also known as CD195; CXCL10, chemokine (C–X–C motif) ligand 10, IP-10; CXCL11, chemokine (C–X–C motif) ligand 11, I-TAC; CXCL9, chemokine (C–X–C motif) ligand 9, Mig; CXCR3, C–X–C chemokine receptor type 3, CD183; DSC3, desmocollin 3; DSP, desmoplakin; DST, dystonin; EDNRB, endothelin receptor B; EDTA, ethylene diamine tetra-acetic acid; EFNB3, ephrin B3; FLG, filaggrin; FZD3, frizzled class receptor 3; IDO, Indoleamine-2,3-dioxygenase; IL-10, interleukin-10; IRF1, interferon regulatory factor 1; JUP, junction plakoglobin; MYC, c-myc; OBM, overexpression of barrier molecule genes; PD-L1, programmed-death ligand 1; PKP3, plakophilin 3; PPL, periplakin; SOX11, SRY (sex determining region Y)-box 11; SOX2, SRY (sex determining region Y)-box 2; TACSTD2, tumor-associated calcium signal transducer 2, trop2; TCF12, transcription factor 12; TCGA, The Cancer Genome Atlas; TGFβ, transforming growth factor β; Th1, T helper type 1; TME, tumor microenvironment; VEGFA, vascular endothelial growth factor A; WNT7B, wnt family member 7B

ARTICLE HISTORY
Received 29 July 2016
Revised 19 September 2016
Accepted 20 September 2016

KEYWORDS
Adherens junction; cancer immunology; desmosome; filaggrin; immune privilege; immunosuppression; melanoma; ovarian cancer; TIL; tumor microenvironment

Introduction

Immune signatures associated with T-cell infiltration of tumors, including melanoma and ovarian cancer, are associated with improved clinical outcomes.[1-11] The mechanisms enabling and regulating T-cell infiltration and function in the tumor microenvironment (TME) are being elucidated. Roles have been established for T-cell homing receptors,[12] chemokine receptors, endothelial molecules,[13,14] decreased antigen presentation and intratumoral molecules that interfere with T-cell function and survival in the TME.[6,15-19] However, little is known about mechanical barriers to lymphocyte infiltration into tumors.

CONTACT Craig L. Slingluff, Jr., M.D cls8h@virginia.edu Division of Surgical Oncology, Department of Surgery, University of Virginia, P.O. Box 800709, Charlottesville, VA 22908-0709, USA.
Dr Salerno is now Assistant Professor of Surgery at the University of Alabama, Birmingham, AL, USA, and Dr Shea is now Chief of Dermatopathology, and a practicing dermatologist at the Hunter Holmes McGuire Veterans Administration Hospital, Richmond, VA, USA; Dr Pinczewski is now a practicing pathologist at Dorevitch Pathology in Australia.

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

In sites of immunologic privilege, including brain, testes and retina, immune protection is achieved through an immunosuppressive signaling milieu in combination with a physical barrier of cell–cell adhesion at the blood interface.[20-25] Intact mechanical barrier function is critical to maintenance of immune privilege in these sites. In the eye, aberrant anti-retinal T cells are prevented from causing autoimmune disease by the mechanical blood-retina barrier protecting the ocular compartment,[20] and both physical barriers and immune regulation mediate ocular immune privilege.[26] Analogously, brain-reactive T cells, abundant in the normal immune repertoire, are prevented from initiating autoimmune encephalitis largely by the tight intercellular junctions of the blood-brain barrier.[27] Similarly, immune privilege of the male testis is explained by a blood-testis barrier which is a complex anatomic and physiologic barrier in which tight junctions and desmosomal proteins play critical roles.[28,29] Thus, in privileged sites, loss of integrity of either the physical immunologic barrier or signaling milieu may interfere with immune privilege.

Although mechanical barrier function at the blood-retina and blood-brain interfaces is achieved primarily through intercellular tight junctions, the blood-testis and skin-external environment barriers are mediated through both tight junctions and desmosomal adhesion. Desmosomes, or macula adherens, are intermediate filament-based cell–cell adhesions using desmosomal cadherins anchored to intermediate filaments via desmoplakins. Desmosomes are reinforced by armadillo proteins including plakophilin. Expression of desmosomal barrier molecules has been observed in several solid tumors, with mixed prognostic associations.[30-37] In melanoma, elevated levels of the cadherin desmocollin 3 (DSC3) has been associated with increased metastatic risk, but in colon and lung cancer, it has been associated with a better prognosis.[31-33] These associations remain to be developed and explained, and relationships between tumor expression of barrier molecules and tumor-infiltrating lymphocytes have not yet been reported. We hypothesized that proteins engaged in mechanical barrier formation may be overexpressed in tumors without T-cell infiltration and may have roles limiting T-cell infiltration. Here, we report a novel relationship observed in subsets of melanoma and ovarian carcinomas, in that elevated expression of mechanical barrier genes are correlated with a lack of immune signature genes.

Results

Melanoma metastases lacking immune gene signatures have elevated barrier molecule gene expression

Gene expression analysis of human melanoma metastases identified three subsets of melanomas: type 1, characterized by immune signatures, and types 2 and 3 both lacking immune signatures.[6] Group 2 tumors clustered more closely to Group 1 but had low expression of most immune signature genes. Group 3 tumors clustered with melanoma cell lines and melanocyte lines. We hypothesized that the absence of immune cell infiltration in group 2 and/or group 3 tumors may be mediated by genes that actively interfere with infiltration. Thus, we screened the 200 genes with greatest variance among these melanoma metastases[6] for genes upregulated in tumors lacking immune cell signatures. Mean gene expression values for each group (1, 2 and 3) were compared to mean values for the remaining two groups. Thus, comparisons were made for group 1 versus groups 2+3, group 2 versus groups 1+3 and group 3 versus groups 1+2. We did not identify significant patterns for group 3 compared to groups 1 and 2 (data not shown), whereas prior work had highlighted increased immune signature genes in group 1 versus groups 2+3.[6] However, there was marked concordant upregulation of a set of genes in group 2 tumors versus groups 1 and 3; this finding was explained by a majority subset of the group 2 tumors (Fig. 1).

Sixty genes had mean expression levels at least 5-fold greater in group 2 than groups 1+3 combined, at a significance of

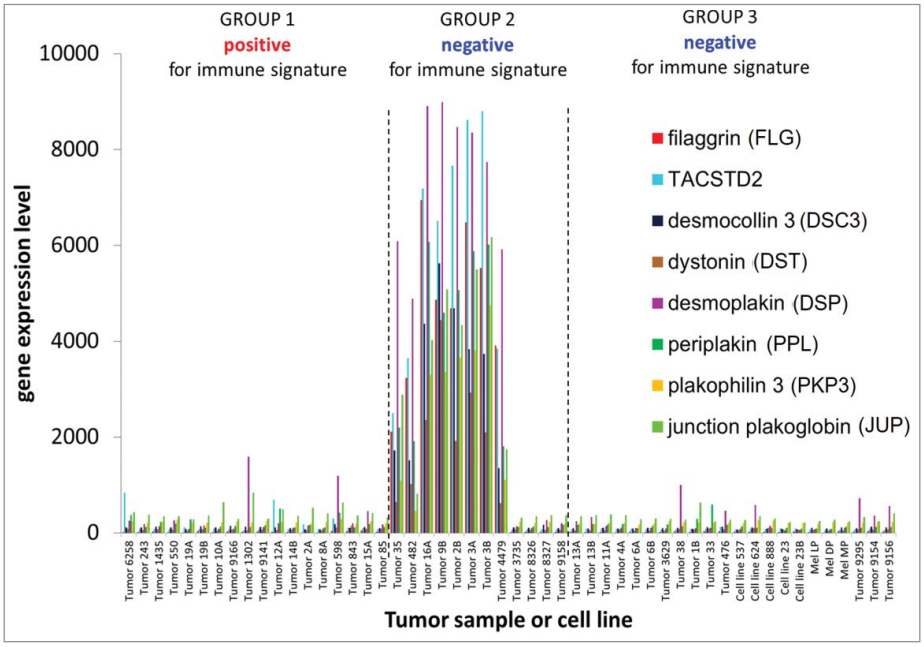

Figure 1. Filaggrin and desmosome-associated gene transcripts in a subset of melanomas lacking immune signature genes.

Table 1. Barrier molecules upregulated in a subset of low-TIL tumors.

Gene (protein)	Fold increase	*p*-value	Protein function
FLG (filaggrin)	131	0.002	Skin cornified envelope formation, flattened keratinocyte morphology
TACSTD2 (tumor-associated calcium signal transducer 2)	32	0.003	Epithelial barrier function, tight junction related proteins. Binds Claudin 1 and 7.
DSC3 (desmocollin 3)	21	0.004	Forms desmosomes
DST (dystonin; bullous pemphigoid antigen 1)	19	0.009	Component of hemidesmosomes
DSP (desmoplakin)	18	0.001	Critical component of desmosomes
PPL (periplakin)	16	0.004	Component of desmosomes and epidermal cornified envelope in keratinocytes
PKP3 (plakophilin 3)	8	0.008	Component of desmosomes, present in nuclei of epithelial cells
JUP (junction plakoglobin)	7	0.005	Participates in intercellular junctions

$p < 0.01$ by two-sided Student's *t*-test for independent samples (data not shown). Among the genes upregulated in group 2 were genes classically associated with cell–cell adhesion and mechanical barrier function, as well as some with known immunologic function. The most upregulated gene was filaggrin (130-fold, $p = 0.002$). It was also notable that five of the nine most upregulated genes (16-fold or higher) were desmosome or tight junction genes, and that two other upregulated genes also encode for desmosomal proteins. These included genes encoding the tight-junction protein tumor-associated calcium signal transducer 2 (TACSTD2, TROP2, 32-fold, $p = 0.003$) and the desmosomal proteins: DSC3 (21-fold, $p = 0.004$), dystonin (DST, bullous pemphigoid antigen 1; 19-fold, $p = 0.009$), desmoplakin (DSP, 18-fold, $p = 0.001$), periplakin (PPL, 16-fold, $p = 0.004$), plakophilin 3 (PKP3, 8-fold, $p = 0.008$) and junctional plakoglobin (JUP, 7-fold, $p = 0.005$). Of 26 tumors lacking immune signature genes (groups 2 and 3), 8 (31%) had elevated barrier molecule gene expression (Table 1 and Fig. 1). Thus, in a subset of melanoma metastases lacking immune gene signatures, there was markedly elevated expression of genes encoding filaggrin and the tight-junction and desmosome-associated proteins listed in Table 1.

Melanoma and ovarian cancer metastases that lack immune signature genes express barrier molecule gene profiles

Having found that filaggrin as well as proteins associated with desmosomes and tight junction are inversely associated with immune gene signatures in a small study of melanoma, we wished to evaluate this observation in a larger and separate set of melanoma metastases and to test this association in a separate epithelial cancer.

Melanoma. For these studies, we utilized gene expression profiling data from a set of 113 metastatic melanomas, which had been collected in a prior study.[38] Two-dimensional self-organizing clustering was performed to examine expression of the eight barrier molecule genes as well as 17 genes comprising a prognostically favorable Th1 immune signature. The genes segregated such that the immune signature genes all clustered together and separately from the barrier molecule genes (Fig. 2A). Among the eight barrier molecules, DST clustered separately from the others (Fig. 2A). A pattern emerged where melanoma metastases with elevated immune signatures lacked barrier molecule expression (right side of Fig. 2A), and a subset of tumors lacking immune signatures had high expression of multiple barrier molecule genes (left side of Fig. 2A). Another subset of melanomas lacked both the immune signature genes and most of the barrier molecule genes; however, these almost always did express high levels of the one barrier molecule DST. There also were a few tumors with selected immune signature genes and barrier molecules; interestingly, these all overexpressed CCR5, IRF1, and usually CXCR3 and its ligands CXCL9-11, with low expression of the other immune signature genes (Fig. 2A, marked with orange bar).

Ovarian cancer. To examine the associations between immune infiltration of tumors and barrier molecule expression in ovarian cancer, gene expression data from 180 advanced ovarian carcinoma specimens were utilized (Fig. 2B).[39] Similar to melanoma tumors, DST again clustered separately from filaggrin and most of the other barrier molecule genes. Perhaps more strikingly than for melanoma, tumors lacking immune signatures were characterized by high expression of barrier molecule genes (left side of Fig. 2B). Specifically, immune signatures were absent in tumors that expressed high levels of at least three barrier molecules (left side of Fig. 2B, section w). Interestingly, high DST expression correlated with a lack of immune signature genes and a lack of expression of other barrier molecules, in ovarian cancer specimens, suggesting that DST may be an effective immune barrier molecule by itself (Fig. 2B, section x), as in melanoma. In total, approximately two out of three ovarian tumors lacking immune signature genes displayed elevated levels of barrier molecule genes. Conversely, tumors with high expression of immune signature genes (right side of Fig. 2B) had relatively low expression of barrier molecule genes.

Correlation between gene expression in melanoma tumor specimens and corresponding cell lines. Additionally, we investigated barrier molecule gene expression correlation between melanoma metastases and their matched cell lines. For correlation analyses, gene expression data were utilized from a subset of 15 melanoma metastases for which matched cell lines were available (Fig. 2C). Among these 15 tumors, there was good concordance of expression of the barrier molecule genes except for DSC3 and DST. Comparing tumors to their matched cell lines, there was poor correlation in gene expression. Interestingly, DST was the only barrier molecule with gene expression that correlated significantly between the melanoma cell lines and tumor metastases ($R = 0.52$; $p = 0.04$, Fig. 2C). The lack of correlation of barrier molecule gene expression between tumor cell lines and tumor metastases suggests that factors in the TME or host may influence the expression of the barrier genes

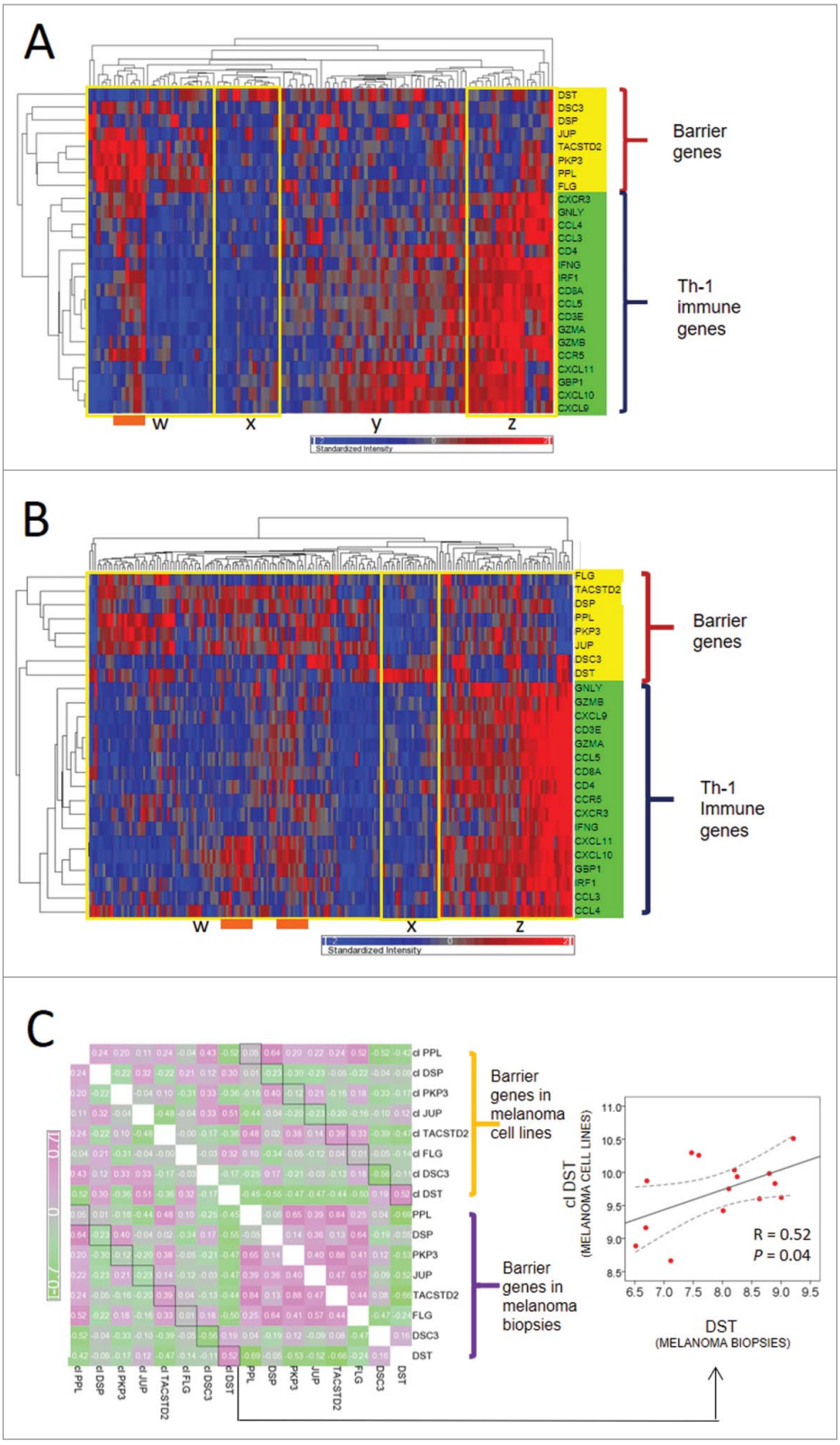

Figure 2. Transcriptional profiling of 113 melanoma metastases and 180 ovarian adenocarcinomas and of melanoma cell lines. Self-organizing heat maps display selected immune signature transcripts together with the expression of mechanical barrier molecules. Genes highlighted in yellow are associated with mechanical barrier function, and in green constitute a Th1 immune signature. For melanoma (A), categories of gene expression are grouped within yellow boxes, from the right, into four subclusters: (z) robust immune signatures, and lacking barrier molecule expression, (y) low or mixed immune signatures and sporadic barrier molecule expression, (x) absent immune signatures with DST overexpression, and (w) overexpression of at least three barrier molecule genes and low level of immune genes. For ovarian cancer (B), yellow boxes mark 3 of the same categories (z, w, x). For both graphs, orange rectangles identify a small subset of tumors in subcluster (w) with high expression of some barrier molecules (but not DST), and limited expression of chemokines and IRF1. (C) Gene–gene matrix correlation between barrier molecule genes in melanoma metastases and their matched cell lines. Gene expression data are from a subset of melanoma metastases (n = 15) for which matched cell lines (n = 15) were available. Each square represents the Pearson product-moment correlation (R), obtained by correlating: (i) genes within tumor metastases, (ii) genes within tumor cell lines or (iii) genes between tumor cell lines and matched tumor metastases. Pink indicates direct correlation, while green inverse correlation. Genes in tumor metastases are labeled with their gene symbol, whereas genes in tumor cell lines are labeled by "cl," followed by their gene symbol.

in vivo. However, the strong correlation of DST expression between melanoma metastases and cell lines suggests that the modulation of this gene may be tumor cell-intrinsic.

Melanoma and ovarian cancer cells express filaggrin and desmosomal proteins

Some of the melanoma tumors evaluated in our gene array studies were from cutaneous or subcutaneous sites. Filaggrin is highly expressed in the epidermis; so a possible explanation for the finding could be inclusion of epidermis in the surgical specimens. However, most cutaneous metastases of melanoma arise in the deep dermis and subcutis, where filaggrin is not expected. Furthermore, one of these metastases arose in lymph nodes and one in small bowel, so it appeared likely that the expression of filaggrin and other barrier molecules could not be explained by epidermal keratinocytes and thus may be from tumor cells themselves. To test whether human melanoma cells directly express filaggrin, we assessed filaggrin expression by immunohistochemistry. Additionally, ovarian cancer specimens were also evaluated to assess expression of filaggrin.

Skin and placental tissue controls revealed intense staining for filaggrin. As expected, in skin, filaggrin expression was confined to the epidermis, and spleen controls lacked filaggrin staining (Fig. 3A). In metastatic melanoma samples, filaggrin expression was varied. We observed examples where filaggrin expression was intense and clearly expressed by tumor cells, and we also found examples of tumors that lacked filaggrin expression (Fig. 3A and B). Similarly, filaggrin expression by ovarian cancer cells was observed in a subset of ovarian cancer specimens (Fig. 3A). These experiments confirm direct tumor cell expression of filaggrin in human melanoma and ovarian carcinoma.

Evaluations of filaggrin expression in melanoma metastases led to an interesting observation that $CD45^+$ immune cell infiltration was inversely correlated with filaggrin expression. In general, tumors with strong filaggrin expression had few infiltrating $CD45^+$ cells, whereas tumors with no detectable filaggrin expression showed diffuse immune cell infiltration (Fig. 3A and B). To evaluate this observation more rigorously, we assessed filaggrin expression in TMAs of melanoma metastases previously evaluated for immune cell infiltration[4]; tumor cores previously noted to contain high (> 300) or low (< 10) $CD8^+$ T cells per core were selected for evaluation of filaggrin protein expression. High or low immune cell infiltration was confirmed by inspection of CD45 staining. For cores with confirmed infiltration phenotype, filaggrin expression was graded from 0 to 3; "high" expression was defined as level 2 or 3 staining (Fig. 3B). Of the tumor cores selected for examination based on past measurements of $CD8^+$ T-cell infiltration,[4] 43 out of 54 (80%) of $CD8^+$-high and 164 out of 199 (82%) of $CD8^+$-low cores were evaluable, representing 21 and 58 tumors, respectively. For each tumor, one to four cores were examined, and heterogeneity of filaggrin expression was noted both among cores from the same tumor deposit, and among cores from different tumors. High filaggrin expression was present in 30% of tumor cores with very low immune infiltrate, but only 9% of cores with high immune infiltrate ($p = 0.007$, Fig. 3C). Thus, these data suggest that filaggrin expression is inversely correlated with $CD8^+$ cell infiltrate in melanoma metastases.

Additionally, melanoma metastases and ovarian cancer specimens were evaluated by immunohistochemistry for direct tumor cell expression of desmosomal proteins, desmoplakin and periplakin. In normal skin controls, desmoplakin and periplakin expression were observed in the epidermis and adnexal structures. A subset of melanoma and ovarian cancer tumors also showed desmoplakin and periplakin expression (Figs. 4A and B), thus indicating that these barrier proteins could be directly expressed by cancer cells. Interestingly, here too we observed a pattern in which melanomas with $CD45^+$ immune cell infiltrate typically lacked expression of the desmosomal proteins, and conversely melanomas with high expression of the desmosomal proteins typically lacked immune cell infiltrates (Figs. 4A and B). Furthermore, this pattern was also observed in ovarian cancer specimens, with $CD45^+$ immune cell infiltrates being inversely correlated with expression of filaggrin and desmosomal proteins (Figs. 4A and B).

Filaggrin, TACSTD2 and desmosomal barrier molecule overexpression is largely independent of endothelin receptor B or WNT/β-catenin overexpression in melanoma and ovarian cancers

Endothelin receptor B (EDNRB) has been reported to interfere with T-cell infiltration into human ovarian cancers,[13,14,16] and activation of WNT/β-catenin signaling has been identified as a mechanism by which melanomas may exclude T cells.[40,41] Thus, we have explored whether EDNRB or WNT/β-catenin overexpression is associated with overexpression of filaggrin, TACSTD2 and desmosomal proteins. Gene expression data were obtained from The Cancer Genome Atlas (TCGA) project and analyzed through cBioPortal.org.[42,43] This data set (accessed 15 May 2016) contains RNA-seq gene expression data from 471 primary and metastatic melanomas. Overexpression of the following genes was assessed ($z > 1.5$): the eight barrier molecules from the present manuscript, EDNRB, nine WNT/β-catenin pathway genes[41] and genes associated with $CD8^+$ T cell infiltration and Th1 immune signatures (CD8A, CD8B, interferon-gamma and CXCL10). Overexpression of Th1 immune genes was identified in 7% of melanomas (Fig. 5A). This number is comparable to the proportion we have identified as having diffuse T cell infiltration (8%), designated as Immunotype C, in a different set of melanoma metastases.[4] Among the melanomas with overexpression of Th1 immune genes, very few overexpressed EDNRB, barrier molecule genes or β-catenin/WNT genes. Among those overexpressing 3–4 of the Th1 genes (Th1-high), 0–1 barrier molecules were overexpressed and there was no overexpression of EDNRB or of β-catenin/WNT genes (Fig. 5C). Even for those overexpressing only 1–2 of the Th1 genes (Th1-low), the vast majority expressed 0–1 of the barrier molecule genes and only 2 overexpressed EDNRB. Thus, high $CD8^+$ T cell infiltration and immune gene signatures are confined to melanomas that do not overexpress the barrier molecules that are the focus of this report, and also that do not overexpress EDNRB or β-catenin/WNT pathway genes.

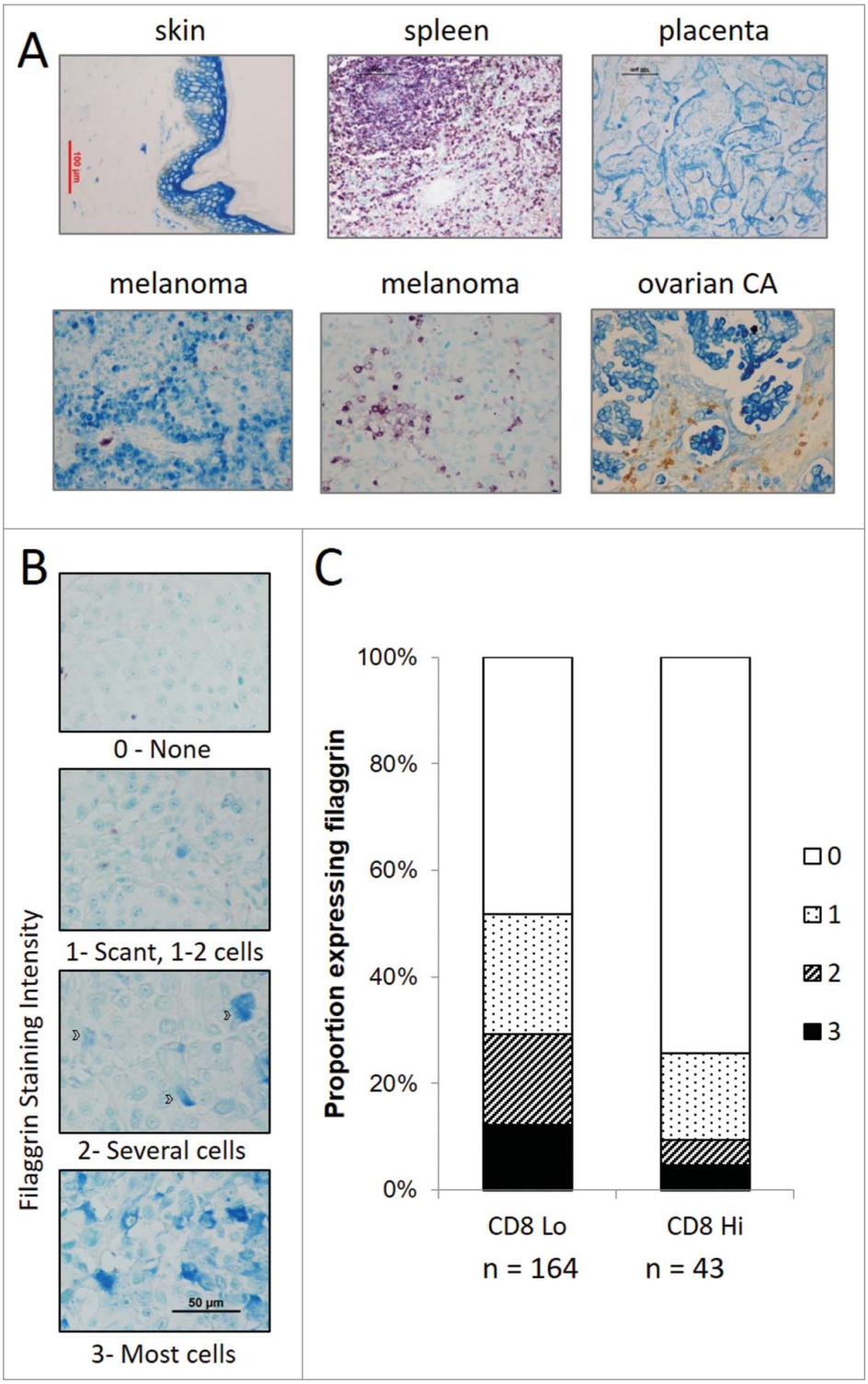

Figure 3. A Filaggrin staining in human tissues: (from top to bottom) normal skin, spleen, placenta, metastatic melanoma, metastatic melanoma and ovarian carcinoma. Specimens are double-stained with filaggrin (blue) and CD45 (purple for spleen, placenta and melanoma, and brown for ovarian cancer); a methyl green counterstain identifies nuclei. (B) Visual analog scale grading filaggrin expression in metastatic melanoma. Scores are based on the number of positively stained cells per 40X field. White arrowheads indicate positively staining cells in levels 1 and 2. (C) Filaggrin staining in melanoma TMA cores with low and high $CD8^+$ infiltration. Levels of staining: 0 (white), 1 (dotted gray), 2 (dark diagonal hash lines), 3 (black), n = number of tumor cores examined.

On the other hand, among 437 melanomas without overexpression of CD8A, CD8B, IFNγ or CXCL10 (non-inflamed), 62% overexpress EDNRB, barrier molecules or WNT/β-catenin genes. One group primarily overexpressed EDNRB, while another had overexpressed barrier molecules (OBM), and a third overexpressed one or more of the WNT/β-catenin genes (Fig. 5A). Among the OBM group, there was striking concordance of overexpression for seven of the eight barrier molecules, while another set of melanomas was identified by overexpression of DST (also known as BPAG1) alone, (Fig. 5A). Overall, 1–7 of the barrier molecule genes were overexpressed in 129 (30%, Fig. 5B). Among these, 44 also

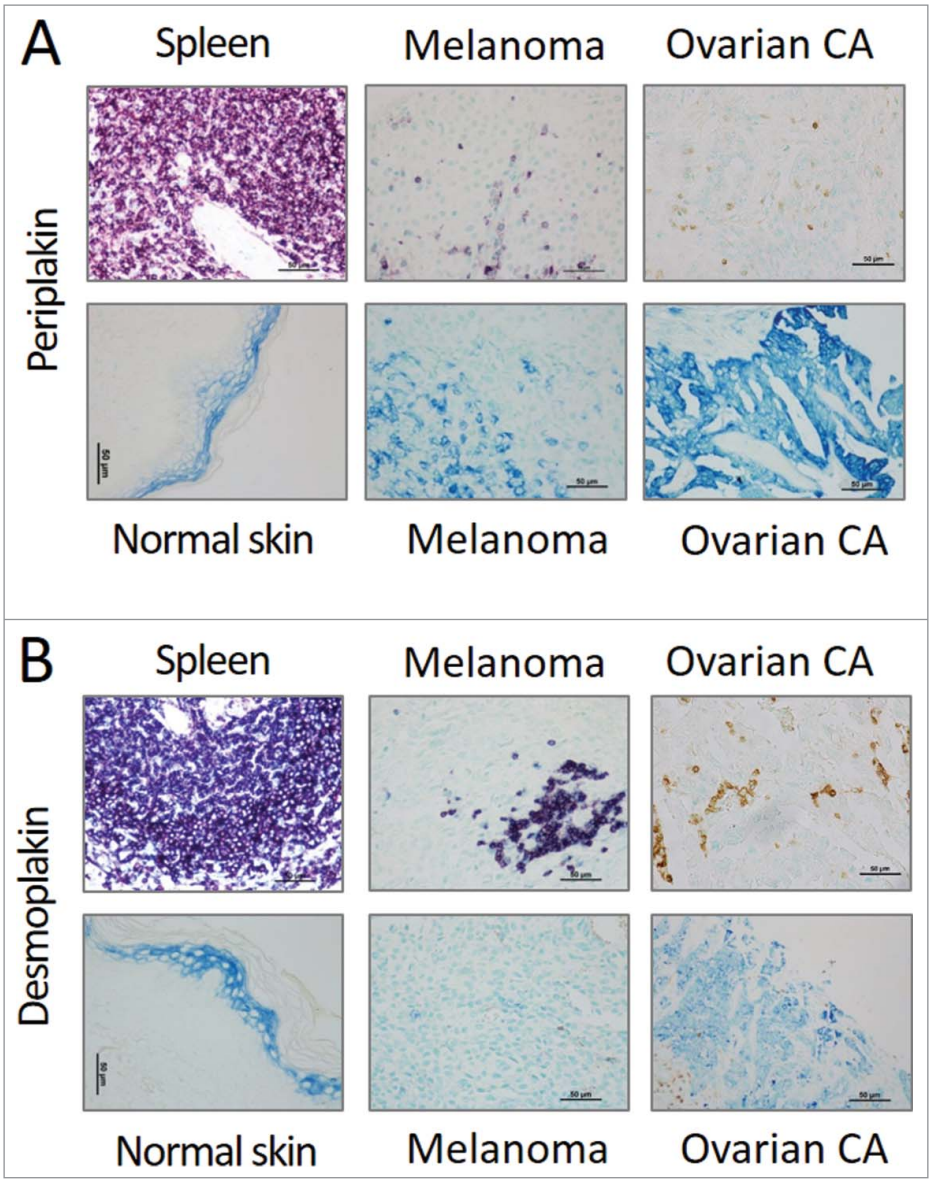

Figure 4. (A) Periplakin (blue) and (B) desmoplakin (blue) staining in melanoma and ovarian carcinoma specimens. Melanoma and ovarian carcinoma specimens are double-stained with CD45 (purple for melanoma, and brown for ovarian CA), and a methyl green counterstain identify nuclei. Tissues controls for each panel are spleen (top left, negative control), and normal skin (bottom left, positive control).

overexpressed one or more WNT/β-catenin genes, 10 also overexpressed EDNRB and 10 overexpressed genes in all three groups. Thus, 15% of the non-inflamed tumors had overexpression of the barrier molecules without overexpression of EDNRB or WNT/β-catenin pathway genes. On the other hand, 22% of the non-inflamed tumors had overexpression only of WNT/β-catenin pathway genes, and 4% had overexpression only of EDNRB (Fig. 5B). Analyses of gene co-occurrence or mutual exclusivity in the TCGA also confirmed significant co-occurrence of genes within each of the groupings (Th1 genes, barrier molecule genes, WNT/β-catenin genes) in melanoma, and mutual exclusivity between members of each grouping, in both melanoma and ovarian cancer (Table S1). Thus, there are subsets of melanomas and ovarian cancers overexpressing the barrier molecule genes (FLG, TACSTD2, DST and five other desmosomal genes) that are distinct from those with other known mechanisms for T-cell exclusion.

Associations between barrier molecule overexpression and patient survival

Immune cell infiltrates in melanoma and ovarian cancer have been associated with significantly prolonged patient survival and may predict response to immune therapies. Thus, we hypothesized that overexpression of barrier molecules would be associated with shorter patient survival. Kaplan–Meier curves are shown in Fig. 6. For the 478 melanoma samples in the TCGA dataset, RNAseq data are available for 471, and survival data are available for 458 (219 deceased, 239 censored). Follow-up times among the deceased and censored groups are similar, with 50% and 51% evaluable to year 3, and 13% and 18% evaluable to year 10, respectively (Table S2). For ovarian cancer, follow-up intervals are somewhat longer for the deceased patients than for the censored patients, with 48% and 34% evaluable to year 3, and 16% and 22% evaluable to year 5, respectively (Table S2).

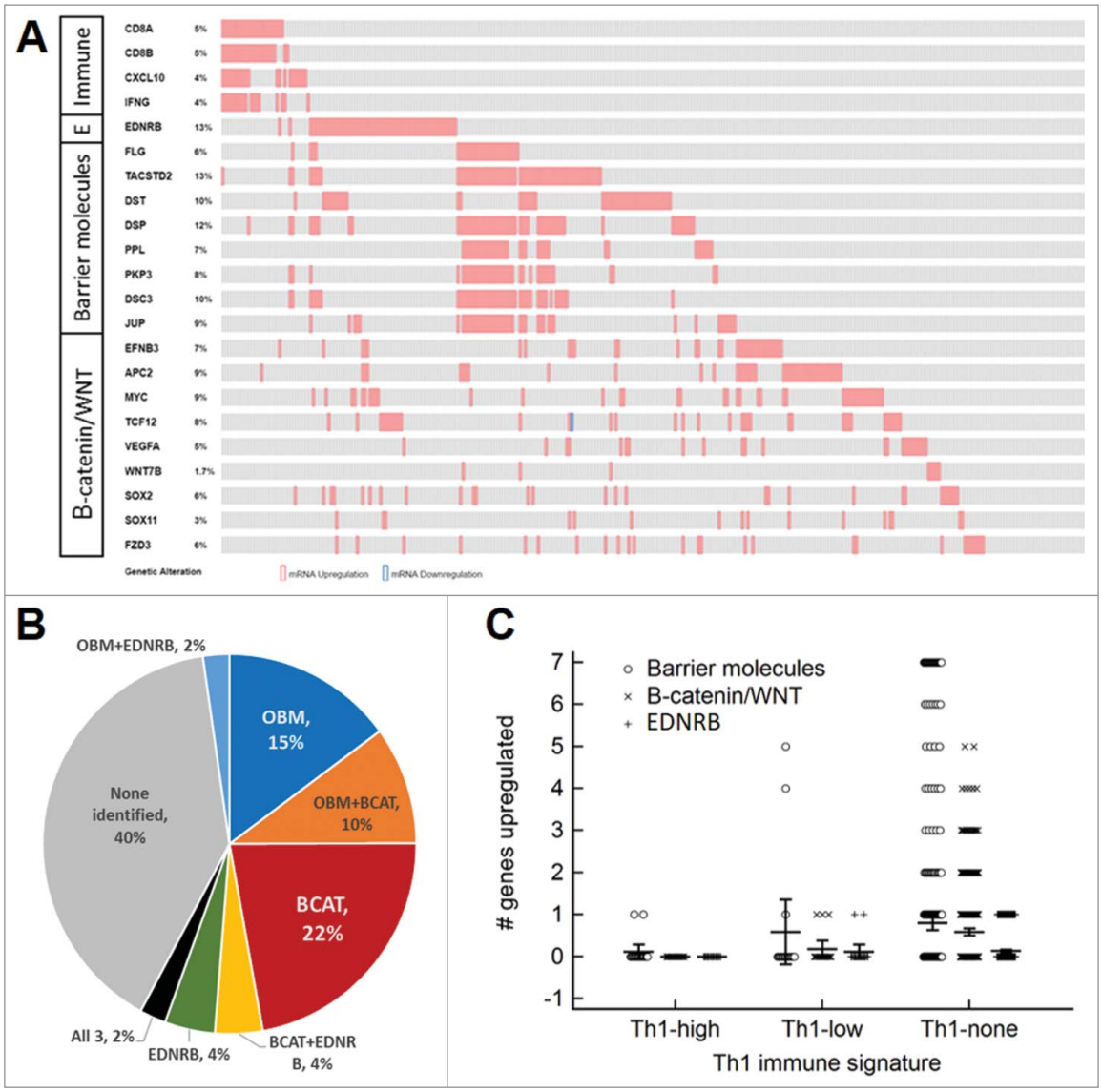

Figure 5. Overexpression of barrier molecules identifies melanomas that lack immune signatures and are largely distinct from those that overexpress endothelin receptor B or WNT/β-catenin genes. (A) Overexpression of genes in each tumor is shown by a red bar ($z > 1.5$), and reduced expression is shown by a blue bar ($z < -1.5$). Th1 immune genes (CD8A, CD8B, IFN-gamma, CXCL10) are grouped at the top (Immune); then ETNBR (E) ; then barrier molecule genes (FLG, TACSTD2, DST, DSP, DSC3, PPL, PKP3, JUP) are grouped in the middle (Barrier molecules), and genes in the β-catenin/WNT pathway (EFNB3, APC2, MYC, TCF12, VEGFA, WNT7B, SOX2, SOX11, FZD3) are grouped at the bottom. This image was obtained from the TCGA bioportal (cbioportal.org); (B) The proportion of 437 "cold" melanomas lacking overexpression of Th1 immune genes in the TCGA database are shown, with overexpression of barrier molecule genes (OBM), β-catenin/WNT genes (BCAT), the endothelin B receptor gene (ETBNR) alone or in combination, as indicated; (C) Tumors were organized into three groups based on the number of Th1-immune genes overexpressed (0 = Th1-none; 1–2 = Th1-low; 3–4 = Th1-high); For each tumor, the number of the eight barrier molecule genes that are overexpressed is represented by a circle, the number of the nine β-catenin/WNT1 genes that are overexpressed is identified by an x, and overexpression of ETNBR is indicated by a plus sign.

For melanomas, there was significantly shorter survival in patients with tumors overexpressing FLG (n = 28, $p = 0.012$, Fig. 6A), or TACSTD2 (n = 61, $p = 0.0002$, Fig. 6B), or five of the remaining barrier molecules (DSC3, DSP, PPL, PKP3, JUP, n = 80, $p < 0.02$, data not shown). However, overexpression of DST was not associated with significantly different survival (n = 47, Fig. 6D). Overexpression of any of the eight barrier molecules was strongly associated with decreased patient survival ($p = 0.0002$, Fig. 6E). On the other hand, survival was not diminished for patients whose melanomas overexpressed EDNRB (Fig. 6E), or any of the WNT/β-catenin genes, individually (p-values = 0.17 to 0.91, data not shown) or in aggregate (Fig. 6F).

Patients whose melanomas overexpressed FLG, TACSTD2 or any of the eight barrier molecules were similar to those without overexpression in terms of age, gender, mutation counts and the incidence of neoadjuvant and adjuvant therapy (Table S3). However, FLG- or TACSTD2-overexpressing tumors (n = 29 and 64, respectively) were less likely to be from metastases and less likely to be stages III and IV, but the primary melanomas for those patients tended to be thicker and were more likely ulcerated than those not overexpressing those barrier molecules. On the other hand, mitotic rate tended to be lower for the FLG- or TACSTD2-overexpressing melanomas. Thus, there were clinical features associated with lower risk on one hand but higher risk on the other hand, These differences were diminished when considering the population of all patients whose melanomas overexpressed at least one of the eight barrier molecules (n = 168, Table S3). To assess whether

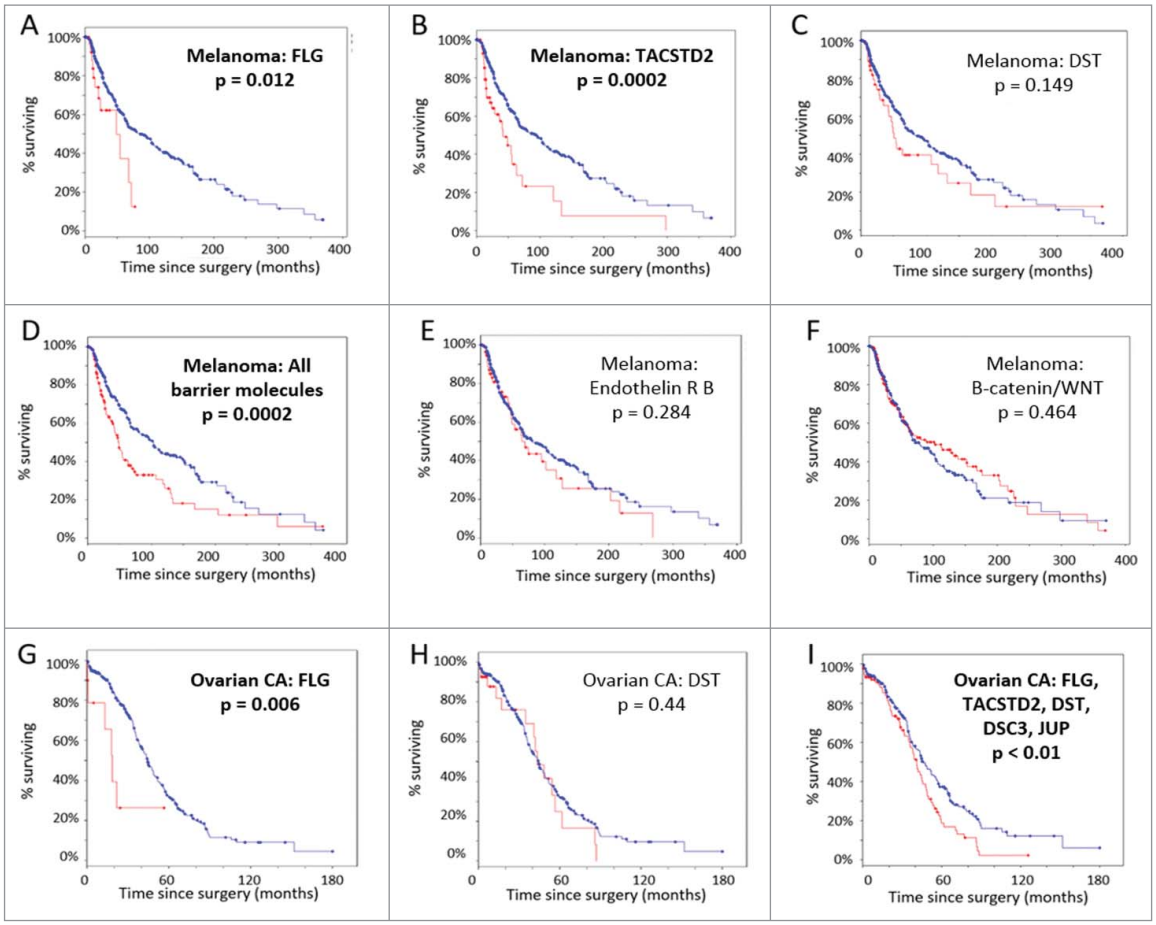

Figure 6. Associations between overall survival and overexpression of barrier molecule genes or β-catenin/WNT1 genes in melanoma or ovarian cancer, in The Cancer Genoma Atlas. Overall survival of patients with melanoma is significantly decreased with overexpression of genes for barrier molecules filaggrin (A), TACSTD2 (B), but not dystonin (C). Survival is significantly decreased for all melanomas overexpressing any of the eight barrier molecules (D). Survival is not associated with overexpression of endothelin receptor B (E) or any of the β-catenin/WNT genes (EFNB3, APC2, MYC, TCF12, VEGFA, WNT7B, SOX2, SOX11, FZD3) in melanoma (F). In ovarian cancer, overexpression of FLG is associated with worse survival (G), whereas DST overexpression is not associated with lower survival (H). On the other hand, expression of one or more of the barrier molecules FLG, TACSTD2, DST, DSC3, JUP was associated with decreased patient survival (I). In each graph, the red line represents the tumors with overexpression of one or more of the selected genes. Overexpression was based on a z score of 1.5 or greater.

the associations of barrier molecule overexpression with shorter patient survival may be attributed to differences in clinical risk factors, those associations were assessed for several relevant clinical subsets. The significant association of overexpression of any of the eight barrier molecules with shorter survival persists within the following subsets of melanoma patients: stages I and II ($p = 0.0013$), M0 melanomas ($p = 0.0086$) and patients who received adjuvant therapy for melanoma ($p = 0.0007$, Table S4). Significant associations did not persist for patient subsets with sample size significantly smaller than the full data set, but significant associations did persist for most of the larger clinical patient subsets also for FLG and TACSTD2 (Table S4). The negative associations with survival persist among patients who received adjuvant therapy, for FLG ($p < 0.05$), TACSTD2 ($p = 0.014$) and for the set of all barrier molecules, which is notable despite the modest subset of 90 patients reported to have received systemic adjuvant therapy (Table S4).

For ovarian cancers, there was significantly shorter survival for patients with tumors overexpressing FLG (n = 10, $p = 0.006$, Fig. 6G), but not DST (n = 27, $p = 0.44$, Fig. 6H). Overexpression of FLG, TACSTD2, DST, DSC3 and/or JUP was evident in 91 (30%), and these patients had significantly poorer survival ($p < 0.01$, Fig. 6I). Overexpression of PPL alone was also associated with poorer survival ($p = 0.026$, not shown), but the remaining two barrier molecules, DSP and PKP3, were not associated with survival differences. Patients whose ovarian cancers overexpressed FLG, or any of the five barrier molecules FLG, TACSTD2, DST, DSC3, JUP were very similar to those without overexpression, in terms of age, gender, mutation counts, tumor size and grade, incidence of vascular invasion, and overall stage (Table S5). For ovarian cancer, the vast majority of tumors (>90 %) were stages III and IV on initial diagnosis, and the tumor specimens themselves were primary tumors in 98% of patients. Significant associations of barrier molecule overexpression persist among patients limited to those dominant subsets, both for FLG overexpression ($p < 0.01$), and for overexpression of any of the eight barrier molecules ($p < 0.02$, Table S6).

Discussion

In sites of immune privilege, immune cell infiltration is limited by multiple barriers. These may include physical or mechanical barriers created by endothelial or epithelial cells with tight

junctions, or functional barriers created by immunosuppressive molecules including IDO, TGFβ, PD-L1 and IL-10.[44] In cancer, the roles of immunosuppressive molecules in the TME are now appreciated as mechanisms of immune escape by tumors, which can otherwise be targeted effectively with immunotherapy.[45,46] Other studies have identified molecular mediators of T-cell exclusion from human cancers, including EDNRB overexpression in human ovarian cancers[14] and genes in the WNT/β-catenin pathway in melanoma.[40,41] Here, we provide new evidence that both melanoma and ovarian cancer cells can express genes encoding proteins with known mechanical barrier function, and that expression of those genes is associated both with the lack of immune gene signatures and with significantly shorter overall patient survival.

We have found that a subset of metastatic melanomas and ovarian carcinomas express high levels of genes encoding eight barrier molecules, including filaggrin, TACSTD2 and six desmosomal proteins. These molecules are classically associated with keratinocytes and skin, especially the most superficial epidermal layer, which is a critical barrier layer. Melanomas and ovarian cancers that are densely infiltrated by T cells have low expression of the barrier molecules, and a subset of those cancers with high expression of the barrier molecule genes usually lack T-cell infiltrates and immune signatures, as is evident across multiple gene expression datasets (Figs. 1, 2 and 5). Most of these barrier molecules (FLG, TACSTD2, DSP, PPL, DSC3, PKP3, JUP) are co-expressed with each other, as is particularly evident in the pilot data set in Fig. 1, and in the larger TCGA dataset in Fig. 5A. Interestingly, another subset of melanomas lacking immune signatures have high expression only of DST, but lack the other barrier molecules (Figs. 2A and B and Fig. 5A). Also, there is a small subset of melanoma metastases (7%, Fig. 2A) and a similar proportion of ovarian cancers (Fig. 2B) that co-overexpress both a subset of immune signature genes and a subset of barrier molecules. Interestingly, these all have very low expression of the barrier molecule DST, and almost all have marked downregulation of IFNγ expression despite IRF1 upregulation. Other than IRF1, the upregulated immune signature genes are limited primarily to CCR5, CXCR3 and GNLY for melanoma and CXCL10 and CXCL11 for ovarian cancers. The TCGA data also identify a very small subset of seven melanomas (< 2% of total) with upregulation of 4–7 of the barrier molecules, but lacking DST, $CD8^+$ and IFNγ overexpression, while also overexpressing one or more Th1 genes (GNLY, CXCL10, CXCL11 and/or CCR5), with a few ovarian cancer patients with similar phenotypes (data not shown). Thus, these data suggest that melanoma and ovarian cancers may be categorized into multiple subgroups based on gene signatures and barrier molecule expression. There is a need to understand the phenotypic and functional correlates of these patterns of immune signatures and barrier molecule expression, and to understand the mechanisms governing immune and barrier function.

We have also evaluated TCGA data for overexpression of EDNRB and of genes in the β-catenin/WNT pathway that have been implicated in T-cell exclusion. Interestingly, these immune exclusion signatures are largely independent. Though some tumors may overexpress both the barrier molecule genes and the β-catenin/WNT genes, or other combinations, 15% of melanomas only overexpress barrier molecule genes, 22% only overexpress β-catenin/WNT genes, and 4% only overexpress endothelin B receptor (Fig. 5B). In particular, barrier molecule genes and β-catenin genes tend to be expressed in a mutually exclusive manner (Table S1). Additional studies may help to clarify the relative contribution of these three gene signatures to T-cell exclusion and what subsets of them may be most useful as prognostic biomarkers.

$CD8^+$ T cell infiltration is associated with improved survival for patients with melanoma or ovarian cancer in this study of TCGA data.[2,4,11,47,48] Since the lack of $CD8^+$ T cell infiltrates is identified by overexpression of barrier molecules, EDNRB or WNT/β-catenin, it would be reasonable to expect that overexpression of any of those gene sets would identify patients with shorter survival. However, we found that overexpression of EDNRB or of WNT/β-catenin genes do not predict decreased survival (Figs. 6E and F). On the other hand, overexpression of the barrier molecules does predict worse survival for melanoma and ovarian cancer (Figs. 6D and I). Also notable is the finding that DST overexpression identifies a subset of cancers with barrier molecule overexpression who lack $CD8^+$ gene signatures (Figs. 2A and B and Fig. 5) but do not have worse survival (Figs. 6C and H). Any large data set can be limited by variations in the data quality and/or biases in the selection of patients and their survival follow-up. However, survival data are available for the vast majority of the patients with RNAseq data, and follow-up intervals for the deceased and censored groups are similar (Fig. S2). The associations of high barrier molecule expression with poor overall survival persist in multiple clinical subsets of patients with melanoma and ovarian cancer (Tables S4 and 6). Interestingly, there is a significant association in melanoma patients who received adjuvant therapy (Table S4), suggesting that the lack of barrier molecule overexpression may have positive predictive value for improved survival after adjuvant therapy, which was primarily high-dose interferon in this era. Thus, there may be value in exploring associations with response to other systemic therapies. Certainly, additional validation of the survival associations in large datasets would be valuable, but within the limitations inherent to this data set, we find that expression of genes FLG, TACSTD2, and the desmosomal genes DSP, DSC3, PKP3, PPL and JUP are unique among other markers of T-cell exclusion by their identification of cancers with decreased survival.

A limitation of the present study is that it does not establish functions of the barrier molecules or a specific mechanism for their association with lack of T-cell infiltration and poorer survival. However, these genes encode proteins with known mechanical barrier function. Filaggrin is normally expressed in the epidermis, and is secreted by keratinocytes to form a polymeric mechanical barrier. Filaggrin expression by cancer cells was not expected or previously reported, but we confirmed expression in melanoma and ovarian cancer cells, by immunohistochemistry (Fig. 3). In melanoma, filaggrin localization appears to be predominantly intracellular and not secreted; thus, further investigation is needed to address how filaggrin may affect T-cell infiltration in tumors. Filaggrin upregulation is associated with overexpression of one or more other intercellular barrier molecules, among them periplakin

and desmoplakin, which may be regulating the formation of desmosomes or other intercellular barrier junctions. By immunohistochemistry, we have confirmed that periplakin and desmoplakin proteins are also expressed in human melanomas and ovarian cancers (Fig. 4). Further studies are needed to define the function of these barrier molecules in cancers. Interestingly, the expression of barrier molecules in melanoma cell lines often is discordant with expression in the surgical tumor specimens from which the lines were derived (Fig. 2C). Thus, expression of the barrier molecules by cancer cells is likely to be modulated by other cells in the TME or by soluble factors. This suggests that expression of barrier molecules may be susceptible to therapeutic modulation once the factors underlying their expression are defined.

New immune and targeted therapies are capable of inducing T-cell infiltration along with tumor control,[49-52] but subsets of tumors fail to respond to these therapies and fail to be infiltrated by immune cells.[53] The present report suggests that there may be several different phenotypes of non-inflamed tumors that lack immune signatures, which may explain different mechanisms for T-cell exclusion and clinical outcome. A better understanding is needed of the mechanical and biochemical barriers to T-cell infiltration, retention and function in the TME. Disruption of these barriers offers the promise of new therapeutic approaches and potential for combined treatments to render more tumors responsive to immune therapy.

Materials and methods

Screening for genes upregulated in tumors without immune signatures

In prior work, gene expression profiling of human melanoma metastases identified three subsets of melanomas: group 1 was characterized by expression of genes for T-cell receptor, immunoglobulin and cytokines (immune signature), and groups 2 and 3 both lacked those immune signatures. Expression levels of the 200 genes with greatest variability across the samples were published in the appendix for that study.[6] We calculated the mean raw intensity of gene expression for each of the listed genes. Means were calculated for each gene across each tumor group, and a fold-increase in expression over the others was calculated. Genes with 5-fold or greater elevation at a significance level of $p < 0.01$ were selected for further study (Table 1).

Gene expression analysis in metastatic melanoma and ovarian carcinoma

Melanoma and ovarian carcinoma samples. Primary snap-frozen ovarian cancer biopsies were collected at the University of Turin (Turin, Italy) from previously untreated patients undergoing debulking surgery after verbal informed consent. Pretreatment snap-frozen metastatic melanoma samples were collected from 113 patients enrolled in five sequential adoptive therapy trials at the National Cancer Institute (NCI), Bethesda, Maryland. All patients signed an informed consent approved by the NCI Institutional Review Board. Fifteen melanoma cell lines derived from melanoma metastases were also analyzed; early passage cultures were used, and clonal sub-selection was not performed.[38] Cell lines were cultured with RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Cellgro), 0.01% L-glutamine Pen-Strep solution (Gemini Bio-Products), 0.001% Ciprofloxacin (10 mg/mL) and 0.01% Fungizone Amphotericin B (Gibco), and detached with 0.2% Trypsin-EDTA (Gemini Bio-Products).

Gene expression assays performed on metastatic melanoma and ovarian carcinoma tumor specimens. Total RNA was extracted with the Qiagen miRNeasy Mini kit and its quality tested with the Agilent Bioanalyzer 2000 (Agilent Technologies, Palo Alto, CA). RNA amplification was performed according to manufacturer's instructions (WT Expression Kit; Ambion, Austin, TX). aRNA were reverse transcribed into cDNAs followed by fragmentation. After hybridization to the GeneChip Human Gene 1.0 ST Arrays, the chips were labeled with a WT Terminal Labeling Kit (Affymetrix, Santa Clara, CA) and scanned on a GeneChip Scanner 3000 7G (Affymetrix). Data were normalized using the Robust Multi-Chip Average (RMA) method and Log2 transformed using Partek Genomics Suite 6.4 (Partek Inc., St. Louis, MO). Data analyses were based on the whole transcripts. Some data from these gene expression studies have been published.[38,39] Self-organizing hierarchical clustering was used to generate heat maps based on the barrier molecules of interest and selected genes associated with prognostically favorable Th1 immune signatures.[54] Cluster analysis was performed using Partek software.

Immunohistochemistry

Tumor tissue microarrays (TMA) of melanoma metastases and ovarian carcinomas were prepared as previously described.[4,11] These and other additional formalin-fixed paraffin-embedded specimens of tissue and tumor specimens were analyzed (UVA IRB numbers 5202, 10598, 13281). Tissue sections were deparaffinized, hydrated using xylene and a graded alcohol series, and antigen retrieval was performed (Vector Laboratories, Burlingame, CA). Sections were stained with antibodies to: filaggrin (Novus Biologicals, Littleton, CO), Periplakin (PPL, Sigma-Aldrich, St. Louis, MO) or desmoplakin (DSP, Progen, Heidelberg, Germany), and detected using an alkaline phosphatase kit and Vector Blue (Vector Laboratories). Double-staining with CD45 antibody (Dako, Carpinteria, CA) was also performed on most sections, and detected using a horseradish peroxidase kit and VIP, Vector Purple, or 3,30-diaminobenzidine (DAB) chromogens (Vector Laboratories). After rinsing with water, sections were counterstained with Hematoxylin, or methyl green, and cover-slipped with mounting medium (Vector Laboratories). Negative control slides were obtained by omitting the primary antibodies.

RNAseq data and patient survival analysis through The Cancer Genome Atlas (TCGA) portal

RNAseq data in the TCGA were accessed through cbioportal.org May 15, 2016. RNA-seq data were available from 471 patients with cutaneous melanoma and 307 ovarian serous cystadenocarcinomas, both from the TCGA Provisional data sets.[42,43] Overexpression of selected genes was identified at

z = 1.5, and associations with overall patient survival were assessed through the Bioportal using Kaplan–Meier curves and significance tested with a log-rank test. p values < 0.05 were considered significant.

Statistical analysis

For the publicly available melanoma gene expression dataset, differences between groups in mean raw intensity of gene expression were evaluated using two-sided Student's t-tests for independent samples. A χ^2 analysis was used to evaluate proportions of high filaggrin expression between tumors with high and low $CD8^+$ T-cell infiltration. Statistical analyses were performed with SPSS Version 21 (IBM, Armonk, NY). The correlation matrix of tumor biopsy and tumor cell line gene expression data was based on the Pearson product-moment correlation (R).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

The melanoma gene expression data were prepared from 113 melanoma metastases generously provided by Dr Steven A. Rosenberg from the Surgery Branch of the National Cancer Institute, Bethesda, MD. Also, the results published here are in part based upon data generated by the TCGA Research Network: http://cancergenome.nih.gov/.

Funding

Funding support was provided by the National Institutes of Health Research Training Grant T32CA163177-01 (PI, Slingluff, Jones; ES), the Intramural Research Program of the National Institutes of Health (DB, FM), the Conquer Cancer Foundation of the American Society of Clinical Oncology (2011 Young Investigator Award to DB), the University of Virginia Rebecca Clay Harris Memorial Fellowship (SS) and the University of Virginia Beirne Carter Center for Immunology Research Fellowship Training Grant T32 AI007496 (JP). This work was also funded in part by the University of Virginia Cancer Center Support Grant NIH/NCI P30 CA44579 (Biorepository and Tissue Procurement Facility).

ORCID

Joel Pinczewski http://orcid.org/0000-0002-7752-9557
Ena Wang http://orcid.org/0000-0001-6606-6510

References

1. Ascierto ML, Kmieciak M, Idowu MO, Manjili R, Zhao Y, Grimes M, Dumur C, Wang E, Ramakrishnan V, Wang XY et al. A signature of immune function genes associated with recurrence-free survival in breast cancer patients. Breast Cancer Res Treat 2012; 131:871-80; PMID:21479927; http://dx.doi.org/10.1007/s10549-011-1470-x
2. Bogunovic D, O'Neill DW, Belitskaya-Levy I, Vacic V, Yu YL, Adams S, Darvishian F, Berman R, Shapiro R, Pavlick AC et al. Immune profile and mitotic index of metastatic melanoma lesions enhance clinical staging in predicting patient survival. Proc Natl Acad Sci U S A 2009; 106:20429-34; PMID:19915147; http://dx.doi.org/10.1073/pnas.0905139106
3. Dieu-Nosjean MC, Antoine M, Danel C, Heudes D, Wislez M, Poulot V, Rabbe N, Laurans L, Tartour E, de Chaisemartin L et al. Long-term survival for patients with non-small-cell lung cancer with intratumoral lymphoid structures. J Clin Oncol 2008; 26:4410-7; PMID:18802153; http://dx.doi.org/10.1200/JCO.2007.15.0284
4. Erdag G, Schaefer JT, Smolkin ME, Deacon DH, Shea SM, Dengel LT, Patterson JW, Slingluff CL, Jr. Immunotype and immunohistologic characteristics of tumor-infiltrating immune cells are associated with clinical outcome in metastatic melanoma. Cancer Res 2012; 72:1070-80; PMID:22266112; http://dx.doi.org/10.1158/0008-5472.CAN-11-3218
5. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C, Tosolini M, Camus M, Berger A, Wind P et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science 2006; 313:1960-4; PMID:17008531; http://dx.doi.org/10.1126/science.1129139
6. Harlin H, Meng Y, Peterson AC, Zha Y, Tretiakova M, Slingluff C, McKee M, Gajewski TF. Chemokine expression in melanoma metastases associated with CD8+ T-cell recruitment. Cancer Res 2009; 69:3077-85; PMID:19293190; http://dx.doi.org/10.1158/0008-5472.CAN-08-2281
7. Mahmoud SM, Paish EC, Powe DG, Macmillan RD, Grainge MJ, Lee AH, Ellis IO, Green AR. Tumor-infiltrating CD8+ lymphocytes predict clinical outcome in breast cancer. J Clin Oncol 2011; 29:1949-55; PMID:21483002; http://dx.doi.org/10.1200/JCO.2010.30.5037
8. Ono M, Tsuda H, Shimizu C, Yamamoto S, Shibata T, Yamamoto H, Hirata T, Yonemori K, Ando M, Tamura K et al. Tumor-infiltrating lymphocytes are correlated with response to neoadjuvant chemotherapy in triple-negative breast cancer. Breast Cancer Res Treat 2012; 132:793-805; PMID:21562709; http://dx.doi.org/10.1007/s10549-011-1554-7
9. Sato E, Olson SH, Ahn J, Bundy B, Nishikawa H, Qian F, Jungbluth AA, Frosina D, Gnjatic S, Ambrosone C et al. Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. Proc Natl Acad Sci U S A 2005; 102:18538-43; PMID:16344461; http://dx.doi.org/10.1073/pnas.0509182102
10. Wang E, Miller LD, Ohnmacht GA, Mocellin S, Perez-Diez A, Petersen D, Zhao Y, Simon R, Powell JI, Asaki E et al. Prospective molecular profiling of melanoma metastases suggests classifiers of immune responsiveness. Cancer Res 2002; 62:3581-6; PMID:12097256
11. Zhang L, Conejo-Garcia JR, Katsaros D, Gimotty PA, Massobrio M, Regnani G, Makrigiannakis A, Gray H, Schlienger K, Liebman MN et al. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. N Engl J Med 2003; 348:203-13; PMID:12529460; http://dx.doi.org/10.1056/NEJMoa020177
12. Brinkman CC, Peske JD, Engelhard VH. Peripheral tissue homing receptor control of naive, effector, and memory CD8 T cell localization in lymphoid and non-lymphoid tissues. Front Immunol 2013; 4:241; PMID:23966998; http://dx.doi.org/10.3389/fimmu.2013.00241
13. Kandalaft LE, Facciabene A, Buckanovich RJ, Coukos G. Endothelin B receptor, a new target in cancer immune therapy. Clin Cancer Res 2009; 15:4521-8; PMID:19567593; http://dx.doi.org/10.1158/1078-0432.CCR-08-0543
14. Buckanovich RJ, Facciabene A, Kim S, Benencia F, Sasaroli D, Balint K, Katsaros D, O'Brien-Jenkins A, Gimotty PA, Coukos G. Endothelin B receptor mediates the endothelial barrier to T cell homing to tumors and disables immune therapy. Nat Med 2008; 14:28-36; PMID:18157142; http://dx.doi.org/10.1038/nm1699
15. Gajewski TF. Failure at the effector phase: immune barriers at the level of the melanoma tumor microenvironment. Clin Cancer Res 2007; 13:5256-61; PMID:17875753; http://dx.doi.org/10.1158/1078-0432.CCR-07-0892
16. Kandalaft LE, Motz GT, Duraiswamy J, Coukos G. Tumor immune surveillance and ovarian cancer: lessons on immune mediated tumor rejection or tolerance. Cancer Metastasis Rev 2011; 30:141-51; PMID:21298574; http://dx.doi.org/10.1007/s10555-011-9289-9
17. Kanterman J, Sade-Feldman M, Baniyash M. New insights into chronic inflammation-induced immunosuppression. Semin Cancer Biol 2012; 22:307-18; PMID:22387003; http://dx.doi.org/10.1016/j.semcancer.2012.02.008
18. Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. Science 2011;

331:1565-70; PMID:21436444; http://dx.doi.org/10.1126/science.1203486
19. Umansky V, Sevko A. Melanoma-induced immunosuppression and its neutralization. Semin Cancer Biol 2012; 22:319-26; PMID:22349515; http://dx.doi.org/10.1016/j.semcancer.2012.02.003
20. Caspi RR. Ocular autoimmunity: the price of privilege? Immunol Rev 2006; 213:23-35; PMID:16972894; http://dx.doi.org/10.1111/j.1600-065X.2006.00439.x
21. Crane IJ, Liversidge J. Mechanisms of leukocyte migration across the blood-retina barrier. Semin Immunopathol 2008; 30:165-77; PMID:18305941; http://dx.doi.org/10.1007/s00281-008-0106-7
22. Doyle TJ, Kaur G, Putrevu SM, Dyson EL, Dyson M, McCunniff WT, Pasham MR, Kim KH, Dufour JM. Immunoprotective properties of primary Sertoli cells in mice: potential functional pathways that confer immune privilege. Biol Reprod 2012; 86:1-14; PMID:21900683; http://dx.doi.org/10.1095/biolreprod.110.089425
23. Smith BE, Braun RE. Germ cell migration across Sertoli cell tight junctions. Science 2012; 338:798-802; PMID:22997133; http://dx.doi.org/10.1126/science.1219969
24. Petty MA, Lo EH. Junctional complexes of the blood-brain barrier: permeability changes in neuroinflammation. Prog Neurobiol 2002; 68:311-23; PMID:12531232; http://dx.doi.org/10.1016/S0301-0082(02)00128-4
25. Roberts TK, Eugenin EA, Lopez L, Romero IA, Weksler BB, Couraud PO, Berman JW. CCL2 disrupts the adherens junction: implications for neuroinflammation. Lab Invest 2012; 92:1213-33; PMID:22641100; http://dx.doi.org/10.1038/labinvest.2012.80
26. Zhou R, Caspi RR. Ocular immune privilege. F1000 Biol Rep 2010; 2:3; PMID:20948803; http://dx.doi.org/10.3410/B2-3
27. Wekerle H. Breaking ignorance: the case of the brain. Curr Top Microbiol Immunol 2006; 305:25-50; PMID:16724799; http://dx.doi.org/10.1007/3-540-29714-6_2
28. Cheng CY, Wong EW, Lie PP, Li MW, Mruk DD, Yan HH, Mok KW, Mannu J, Mathur PP, Lui WY et al. Regulation of blood-testis barrier dynamics by desmosome, gap junction, hemidesmosome and polarity proteins: An unexpected turn of events. Spermatogenesis 2011; 1:105-15; PMID:22319658; http://dx.doi.org/10.4161/spmg.1.2.15745
29. Cheng CY, Mruk DD. The blood-testis barrier and its implications for male contraception. Pharmacol Rev 2012; 64:16-64; PMID:22039149; http://dx.doi.org/10.1124/pr.110.002790
30. Shimbo T, Tanemura A, Yamazaki T, Tamai K, Katayama I, Kaneda Y. Serum anti-BPAG1 auto-antibody is a novel marker for human melanoma. PLoS One 2010; 5:e10566; PMID:20479946; http://dx.doi.org/10.1371/journal.pone.0010566
31. Cui T, Chen Y, Yang L, Knosel T, Huber O, Pacyna-Gengelbach M, Petersen I. The p53 target gene desmocollin 3 acts as a novel tumor suppressor through inhibiting EGFR/ERK pathway in human lung cancer. Carcinogenesis 2012; 33:2326-33; PMID:22941060; http://dx.doi.org/10.1093/carcin/bgs273
32. Cui T, Chen Y, Yang L, Knosel T, Zoller K, Huber O, Petersen I. DSC3 expression is regulated by p53, and methylation of DSC3 DNA is a prognostic marker in human colorectal cancer. Br J Cancer 2011; 104:1013-9; PMID:21364582; http://dx.doi.org/10.1038/bjc.2011.28
33. Rezze GG, Fregnani JH, Duprat J, Landman G. Cell adhesion and communication proteins are differentially expressed in melanoma progression model. Hum Pathol 2011; 42:409-18; PMID:21193224; http://dx.doi.org/10.1016/j.humpath.2010.09.004
34. Cho EY, Choi Y, Chae SW, Sohn JH, Ahn GH. Immunohistochemical study of the expression of adhesion molecules in ovarian serous neoplasms. Pathol Int 2006; 56:62-70; PMID:16445817; http://dx.doi.org/10.1111/j.1440-1827.2006.01925.x
35. Maynadier M, Chambon M, Basile I, Gleizes M, Nirde P, Gary-Bobo M, Garcia M. Estrogens promote cell-cell adhesion of normal and malignant mammary cells through increased desmosome formation. Mol Cell Endocrinol 2012; 364:126-33; PMID:22963885; http://dx.doi.org/10.1016/j.mce.2012.08.016
36. Furukawa C, Daigo Y, Ishikawa N, Kato T, Ito T, Tsuchiya E, Sone S, Nakamura Y. Plakophilin 3 oncogene as prognostic marker and therapeutic target for lung cancer. Cancer Res 2005; 65:7102-10; PMID:16103059; http://dx.doi.org/10.1158/0008-5472.CAN-04-1877
37. Demirag GG, Sullu Y, Yucel I. Expression of Plakophilins (PKP1, PKP2, and PKP3) in breast cancers. Med Oncol 2012; 29:1518-22; PMID:21947748; http://dx.doi.org/10.1007/s12032-011-0071-1
38. Spivey TL, De Giorgi V, Zhao Y, Bedognetti D, Pos Z, Liu Q, Tomei S, Ascierto ML, Uccellini L, Reinboth J et al. The stable traits of melanoma genetics: an alternate approach to target discovery. BMC Genomics 2012; 13:156; PMID:22537248; http://dx.doi.org/10.1186/1471-2164-13-156
39. Zsiros E, Duttagupta P, Dangaj D, Li H, Frank R, Garrabrant T, Hagemann IS, Levine BL, June CH, Zhang L et al. The ovarian cancer chemokine landscape is conducive to homing of vaccine-primed and CD3/CD28-costimulated T cells prepared for adoptive therapy. Clin Cancer Res 2015; 21:2840-50; PMID:25712684; http://dx.doi.org/10.1158/1078-0432.CCR-14-2777
40. Spranger S, Gajewski TF. A new paradigm for tumor immune escape: β-catenin-driven immune exclusion. J Immunother Cancer 2015; 3:43; PMID:26380088; http://dx.doi.org/10.1186/s40425-015-0089-6
41. Spranger S, Bao R, Gajewski TF. Melanoma-intrinsic β-catenin signalling prevents anti-tumour immunity. Nature 2015; 523:231-5; PMID:25970248; http://dx.doi.org/10.1038/nature14404
42. Cerami E, Gao J, Dogrusoz U, Gross BE, Sumer SO, Aksoy BA, Jacobsen A, Byrne CJ, Heuer ML, Larsson E et al. The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. Cancer Discov 2012; 2:401-4; PMID:22588877; http://dx.doi.org/10.1158/2159-8290.CD-12-0095
43. Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, Sun Y, Jacobsen A, Sinha R, Larsson E et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. Sci Signal 2013; 6:pl1; PMID:23550210; http://dx.doi.org/10.1126/scisignal.2004088
44. Shechter R, London A, Schwartz M. Orchestrated leukocyte recruitment to immune-privileged sites: absolute barriers versus educational gates. Nat Rev Immunol 2013; 13:206-18; PMID:23435332; http://dx.doi.org/10.1038/nri3391
45. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, Powderly JD, Carvajal RD, Sosman JA, Atkins MB et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N Eng J Med 2012; 366:2443-54; PMID:22658127; http://dx.doi.org/10.1056/NEJMoa1200690
46. Munn DH. Blocking IDO activity to enhance anti-tumor immunity. Front Biosci (Elite Ed) 2012; 4:734-45; PMID:22201909; http://dx.doi.org/10.2741/e414
47. Spatz A, Gimotty PA, COOK MG, Van Den Oord JJ, Desai N, Eggermont AM, Keilholz U, Ruiter DJ, Mihm MC. Protective effect of a brisk tumor infiltrating lymphocyte infiltrate in melanoma: An EORTC melanoma group study. J Clin Oncol 2007; 25:8519; PMID:17369575; http://dx.doi.org/10.1200/JCO.2006.08.1463
48. Adams SF, Levine DA, Cadungog MG, Hammond R, Facciabene A, Olvera N, Rubin SC, Boyd J, Gimotty PA, Coukos G. Intraepithelial T cells and tumor proliferation: impact on the benefit from surgical cytoreduction in advanced serous ovarian cancer. Cancer 2009; 115:2891-902; PMID:19472394; http://dx.doi.org/10.1002/cncr.24317
49. Boni A, Cogdill AP, Dang P, Udayakumar D, Njauw CN, Sloss CM, Ferrone CR, Flaherty KT, Lawrence DP, Fisher DE et al. Selective BRAFV600E inhibition enhances T-cell recognition of melanoma without affecting lymphocyte function. Cancer Res 2010; 70:5213-9; PMID:20551059; http://dx.doi.org/10.1158/0008-5472.CAN-10-0118
50. Peng W, Liu C, Xu C, Lou Y, Chen J, Yang Y, Yagita H, Overwijk WW, Lizee G, Radvanyi L et al. PD-1 blockade enhances T-cell migration to tumors by elevating IFN-gamma inducible chemokines. Cancer Res 2012; 72:5209-18; PMID:22915761; http://dx.doi.org/10.1158/0008-5472.CAN-12-1187
51. Ribas A, Comin-Anduix B, Economou JS, Donahue TR, de la Rocha P, Morris LF, Jalil J, Dissette VB, Shintaku IP, Glaspy JA et al. Intratumoral immune cell infiltrates, FoxP3, and indoleamine 2,3-dioxygenase in patients with melanoma undergoing CTLA4 blockade. Clin Cancer Res 2009; 15:390-9; PMID:19118070; http://dx.doi.org/10.1158/1078-0432.CCR-08-0783

52. Wilmott JS, Long GV, Howle JR, Haydu LE, Sharma RN, Thompson JF, Kefford RF, Hersey P, Scolyer RA. Selective BRAF inhibitors induce marked T-cell infiltration into human metastatic melanoma. Clin Cancer Res 2012; 18:1386-94; PMID:22156613; http://dx.doi.org/10.1158/1078-0432.CCR-11-2479
53. Herbst RS, Soria JC, Kowanetz M, Fine GD, Hamid O, Gordon MS, Sosman JA, McDermott DF, Powderly JD, Gettinger SN et al. Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. Nature 2014; 515:563-7; PMID:25428504; http://dx.doi.org/10.1038/nature14011
54. Galon J, Angell HK, Bedognetti D, Marincola FM. The continuum of cancer immunosurveillance: prognostic, predictive, and mechanistic signatures. Immunity 2013; 39:11-26; PMID:23890060; http://dx.doi.org/10.1016/j.immuni.2013.07.008