

Pregnancy Specific Glycoproteins: A Possible Mediator of Immune Tolerance of Cancers

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Received date: January 28, 2021, **Accepted date:** March 19, 2021

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Abstract

Cancer immunotherapy relies upon the immune system recognizing and killing cancer cells. Tumors can elude recognition by readapting existing mechanisms of immune control and suppression. Here we explore the hypothesis that cancers repurpose the immune suppression employed during pregnancy to protect the allogeneic fetus. Those mechanisms are reviewed and shown to be employed both in pregnancy and by tumors. Pregnancy specific glycoproteins (PSGs) produced by fetal trophoblasts are also synthesized by a large number of tumors, which are associated with a poor overall survival of the patient. The family of PSGs may well be a useful target for future checkpoint therapy.

Keywords: Pregnancy-specific glycoproteins; T-cell tolerization; Checkpoint therapy

Introduction

By the 1960's there were a number of suggestions that the immune system played a role in detecting and rejecting cancerous cells [1,2]. The next thirty to forty years saw a large number of attempts to identify the antigens found in a tumor that could lead to tumor rejection. By the 1990's and into the twenty-first century, it became clear that whatever the antigens were, the tumor was being protected from tissue rejection by proteins that tolerized T-cells, such as CTLA-4 [3] and PD-1 or PD-L1 [4]. Antibodies directed against these tolerance factors have been shown to aid in tumor rejection. That this was not the entire story, however, became clear from the observation that these antibodies functioned to help reject tumors only 20-30% of the time in a wide variety of tumor types [5].

Tumors do not invent new mechanisms to hide from the immune system, but rather repurpose existing mechanisms of tolerance, so other negative regulators of T-cell tissue rejection are being explored. Hundreds of clinical trials carried out over the past few years have not found additional mechanisms that hide tumors from the

immune system comparable to PD-1 or CTLA-4. It has been suggested that viviparous pregnancies might well employ processes similar to the evasion of recognition and rejection of tumors by the immune system [6]. Paternal alloantigens can be recognized by the immune system and in some cases may cause rejection of the fetus or immune mediated diseases in the fetus. A comparison of immune tolerance of a fetus and a tumor demonstrates some similarities. The trophoblasts, which derive from the outer cells of the blastocyst, and form the membranes that interface with the placenta, do not express the HLA-A, B or Dr receptors that bind the peptide antigens engaged by CD-8 T-cell receptors, and the loss of one or both alleles of HLA molecules are observed in some tumors [7,8]. HLA G and E receptors are expressed in a variety of tumors and have prognostic utility to indicate unfavorable outcomes for the patient [9]. These HLA types are expressed on trophoblastic cells and bind antigens that result in the tolerance of T-cells and some types of NK cells [10]. CD-4 regulatory T-cells (Foxp3+) that suppress the CD-8 T-cell responses are prominent both in pregnancy and in tumor protection from the immune system [6]. LIF (leukemia inhibitory factor) is a hormone produced by uterine cells

that is required for implantation of the blastocyst in the uterus [11]. The transcription factor that transcribes the LIF gene in the uterus is p53 [11]. LIF, along with progesterone, estradiol, and prostaglandin D2, results in an inversion of the ratio of CD-4 Th-1/Th-2 cell numbers, enhancing B-cell responses but lowering T-cell activity. LIF is produced in a variety of tumors [6]. The cytotrophoblast cells making up the membranes of the embryo and interacting with the placental cells synthesize and secrete ten different pregnancy specific glycoproteins (named PSG-1 to PSG-9 and PSG-11) [12]. Antibodies directed against two of these proteins in a pregnant mouse with an allogeneic fetus resulted in an abortion of the embryos [13]. In humans, secreted PSGs interact with monocytes and macrophages that then secrete, from preformed vesicles, TGF-beta and IL-10, which act upon CD-4 cells to induce CD-4 regulatory T-cell (FoxP3 +). These T-cells express CTLA -4 and PD-1 [14,15]. Some tumors expressing PSGs appear to have a worse outcome than the same types of tumors that do not express PSGs [16,17].

The hypothesis to be explored in this work is that cancers take advantage of the programmed immunosuppression observed in pregnancies that permits allogeneic fetuses and embryos expressing the fathers' antigens to escape immune rejection. This perspective explores the frequency of tumor expression of the PSG proteins in a wide variety of human tumors from the TCGA collection and demonstrates that while the PSGs are in the main coordinately expressed, some PSG proteins play a more prominent role in these tumors than others. It demonstrates that the expression of PSGs in a tumor leads to poorer overall survival of the patient and that the different PSGs, either separately or together, lead to this outcome. Employing RNA sequencing of tumors that produce PSGs indicates that the tissue specificity of the tumor has an impact upon the nature of the infiltrating cell types in these tumors. Based upon these results, antibodies directed against the PSGs ought to be useful in treating the tumors producing them so as to initiate checkpoint therapy and possible tumor rejection.

The Innate and Adaptive Immune System during Pregnancy

The hematopoietic stem cells differentiate into a lymphoid lineage in the adult human bone marrow, which then divides into the progenitors of B-cells that remain for development in the bone marrow, and T-cells that migrate, in late fetal life and for several years after birth, to the thymus. In the thymus, T-cell (CD-3 positive cells) development progresses by producing the beta and alpha chains for T-cell receptors, by recombination between a V-gene (variable), D-gene (diversity), J gene (joining) and C-gene (constant) for each T-cell, followed by additional diversity being created by the enzyme terminal transferase, which gives rise to additional nucleotides in the hypervariable

region of alpha and beta chains. During this development there is positive and negative selection upon each T-cell, with a different receptor under the direction of the HLA-type receptor genes for antigens, reducing or eliminating clones of T-cells that react with self-antigen peptides. Late in this developmental process, the CD-3 positive T-cells are differentiated into CD-4 helper cells and CD-8 killer cells. The human adult has an estimated 10^8 to 10^{10} T-cells that can produce approximately 10^{12} to 10^{14} different combinations of receptors. Not every possible receptor is produced in a person, and even identical twins have different repertoires. In addition, the different receptor sequences are not produced in equal amounts. Some T-cell receptors are produced in independent T-cell clones many times and others only once, so the distributions of receptors are not equal in the newly developed T-cell repertoire (before selection). The T-cell receptors combine with peptide antigens of about 9 amino acids in length (CD-8 cells) to 20-26 amino acids in length (CD-4 cells). These peptides are degraded by polymorphic proteases in the cell from the normal cellular proteins, proteins with mutations (as from cancerous tissue) or foreign proteins of viruses or bacteria. These peptides are loaded onto class 1 receptors for engagement by CD-8 killer cells, or class 2 receptors for engagement by CD-4 cells. The class 1 receptors are encoded by the highly polymorphic HLA-A, B, and C genes and the class 2 receptors are encoded by the highly polymorphic HLA-Dr genes. These receptors bind and present the antigens to the T-cell receptors. Table 1 summarizes four different CD-4 T-cell types that act as helpers to produce antibodies by B-cells (extracellular parasites), activate killer T-cells (intracellular parasites), act as suppressors of T-cells (T-regs) or initiate inflammatory reactions (Th-17 T-cells) and the formation of the inflammasome in response to tissue damage, fighting infections, or producing autoimmunity. Each of these four cell types is induced to differentiate by an interleukin or a hormone, and when mature and engaging an antigen each of these cell types functions by secreting interleukins or cytokines that permit antibody production and class switching, activate a T-cell for killing target cells, prevent T-cells from functioning, or initiate inflammation.

During pregnancy there are some important changes in the activities of these T-cells. Starting during the first trimester, but increasing dramatically in the second and third trimesters, there is an inversion of CD-4 Th-1 and CD-4 Th-2 cell numbers and activity. Cellular cytotoxicity by CD-8 cells declines and there is relative focus upon antibody production. This shift in Th-1/Th-2 ratio (Th-1 cells decrease and Th-2 cells increase) is induced by progesterone, estradiol, prostaglandin D-2, and LIF (leukemia inhibitory factor). The Th-2 cytokines IL-4, IL-6, IL-10 and M-CSF all aid antibody production to fight infections. T-regs, the suppressors of immunity,

| | CELLS | INDUCED BY | SECRETE | OUTCOME |
|----|-----------------|------------------------|-----------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| 1. | Th-1 (CD-8) | IL-12 | IL-2, IFN-G | Cytotoxicity decreased in pregnancy |
| 2. | Th-2 (B- cells) | IL-4, IL-10 | Antibody; IL-4, 5, 10 | Activity increased in 2 nd , 3 rd trimester |
| 3. | T-regs | TGF-beta | Lower immune system (IL-10) | Activity increased in maternal/fetal interface, FoxP3, CD-3 Requires antigenic stimulation from trophoblasts, T-reg memory cells Persists post-pregnancy – along with fetal cells T-regs express CTLA-4, PD-1 |
| 4. | Th-17 | IL-17, IL-1 beta, IL-6 | IL-17 | Pro-inflammatory decreased in pregnancy |

Table 1: CD-4 T-Cells.

increase in number and activity. These cells are observed in large numbers at the placental fetal interface and in the peripheral blood, and there are indications that the cells in these two locations may differ in their activities. T-regs, like all T-cells, require activation by foreign antigens to be suppressors, and they recognize antigens presented by trophoblasts, which are derived from embryonic cells, forming the membranes around the embryo and interfacing with the placenta. HLA-C regulated memory T-regs persist postpartum along with fetal cells that can be detected in the mother years after giving birth. There is a decrease in IL-17 pro-inflammatory T-cells during pregnancy [6].

The placental–uterine interface forms a physical barrier between parts of the mother’s immune system and the fetus. On the surface of the trophoblast cells, which are of fetal origin, the HLA-A and B gene class 1 receptors and the HLA class Dr gene, class 2 receptors are not expressed. These are the major antigen presenting receptors for CD-8 killer cell engagement. However, the HLA-C gene class 1 receptor and the minor HLA-E, F, and G gene class 2 receptors are expressed, but are much more limited in their presentation of antigens to killer T-cells. The HLA-G gene is expressed by fetal derived cells adjacent to the placenta and inhibits immune responses directed against fetal cells. None the less, memory T-cells dependent upon presentation of antigens by HLA-C class 1 receptors persist well after the pregnancy is completed and can detect antigens from a second pregnancy attributable to the same father.

LIF is a hormone secreted by the uterus as a necessary part of implantation of the blastocyst. Its secretion is essential for implantation. Surprisingly, one of the key transcription factors that produce LIF m-RNA in the uterus is p53. P53 knock-out female mice have small litter sizes, but a knock-out deletion in the male has no impact on litter size [11]. LIF acts to inhibit Th-1 CD-4 T-cells,

which promote CD-8 class 1 killing of foreign tissues. LIF also promotes an increased activity of TH-2 CD-4 cells and antibody production by B-cells.

In humans, fertilization occurs in the upper third of the fallopian tubes and implantation of the blastocyst occurs 6-12 days after fertilization. The trophoblasts that surround the surface of the blastocyst divide to produce the membranes that interface with the uterine tissue and the placenta is produced over the next weeks. As early as the third day after fertilization the trophoblasts that surround the blastocyst begin the secretion of a group of glycoproteins called pregnancy specific glycoproteins (PSGs). Their production increases with time so that very large levels of these PSGs are being made in the second and third trimester. The genes that encode the PSGs form a cluster of ten genes and one pseudogene called PSG 1-9 and 11, with PSG-10 being the pseudogene. This cluster of genes maps to chromosome 19q 13.1 to 19q 13.3. The PSGs 1, 6, 9 and 11 secreted glycoproteins act upon monocytes and macrophages to activate a latent form of TGF beta and IL-10 stored in vesicles in these cells, which are then secreted [14,15]. Nothing is known about the PSG receptors (if they exist) that may be on monocytes. TGF beta and IL-10 induce T-regs. These T-regs express CTLA-4 and PD-1, both of which prevent the rejection of foreign tissues by CD- 8 T-cells [14,15]. Pregnant mice that carry allogeneic embryos will abort these embryos when anti-PSG-1 antibodies are administered to them [13].

During pregnancy there is a strong diminution of the T-cell CD-8 ability to reject foreign tissues and an enhancement of the B-cell response. This is brought about by an inversion of the CD-4 Th-1 to Th-2 helper ratio, the loss of HLA-A, B and D expression and diminished Class 1 and 2 presentation of foreign antigens. The increase in T-regs, which suppress the CD-8 T cell responses, is brought about, at least in part, by the PSGs [14,15]. The

production of LIF brings about an enhanced B cell response. It appears that the B-cell response is kept intact so as to help defend against viruses via neutralization of infectivity and bacteria by coating bacteria to enhance macrophage engulfment. The diminution of the CD-8 T-cell response not only protects the embryo from tissue rejection but also moderates autoimmune disease symptoms in the pregnant women.

This is likely an incomplete description of the immune system in pregnant females. There is obviously a programmed response to pregnancy that carries out this change in immunity in females and it seems likely that it is at least in part responsible for the observed sexual dimorphism of the immune system. What mediates these sexual differences is unclear, but both hormonal differences and the genetic inequality of two X chromosomes versus one X chromosome are good starting places to help explain this difference. There is also sexual dimorphism in epigenetic silencing of different loci in the genome that might contribute to this mechanism. The question now becomes: have cancerous tissues adopted some of these same processes in males and females and how much of it can be adopted in males? To what degree is the female programmed immune system responsible for the higher incidence of cancer in males, an earlier onset of cancer in

males, shorter lifespan of males? [6]. And in a reciprocal fashion, a higher incidence of autoimmune diseases in females and a better response to infectious diseases in females? [6]. Or even, why checkpoint therapy directed against eliminating CTLA-4 or PD-1 with antibodies functions better in men than women? [18].

PSG Gene Expression is Associated with Worse Survival Across Tumors

To evaluate the impact of PSG gene family expression across different tumor types, The Cancer Genome Atlas (TCGA) collection, comprising over 10,000 tumors across 33 cancer types, was employed. Compared to normal tissues where expression of these genes is rare, we found upregulation of PSG genes in a majority of tumors, including kidney chromophobe carcinoma (85%), thyroid carcinoma (74%), adrenocortical carcinoma (73%), head and neck squamous cell carcinoma (68%), cholangiocarcinoma (67%), among many others (Figure 1A). Comparison of the RNA expression across the different PSG genes in TCGA tumors indicates that each PSG is upregulated in at least 10% of all tumors, with PSG-9 being the most commonly upregulated of the genes in nearly 25% of TCGA tumors (Figure 1B). These results indicate that upregulation of PSG genes is common across different tumor types.

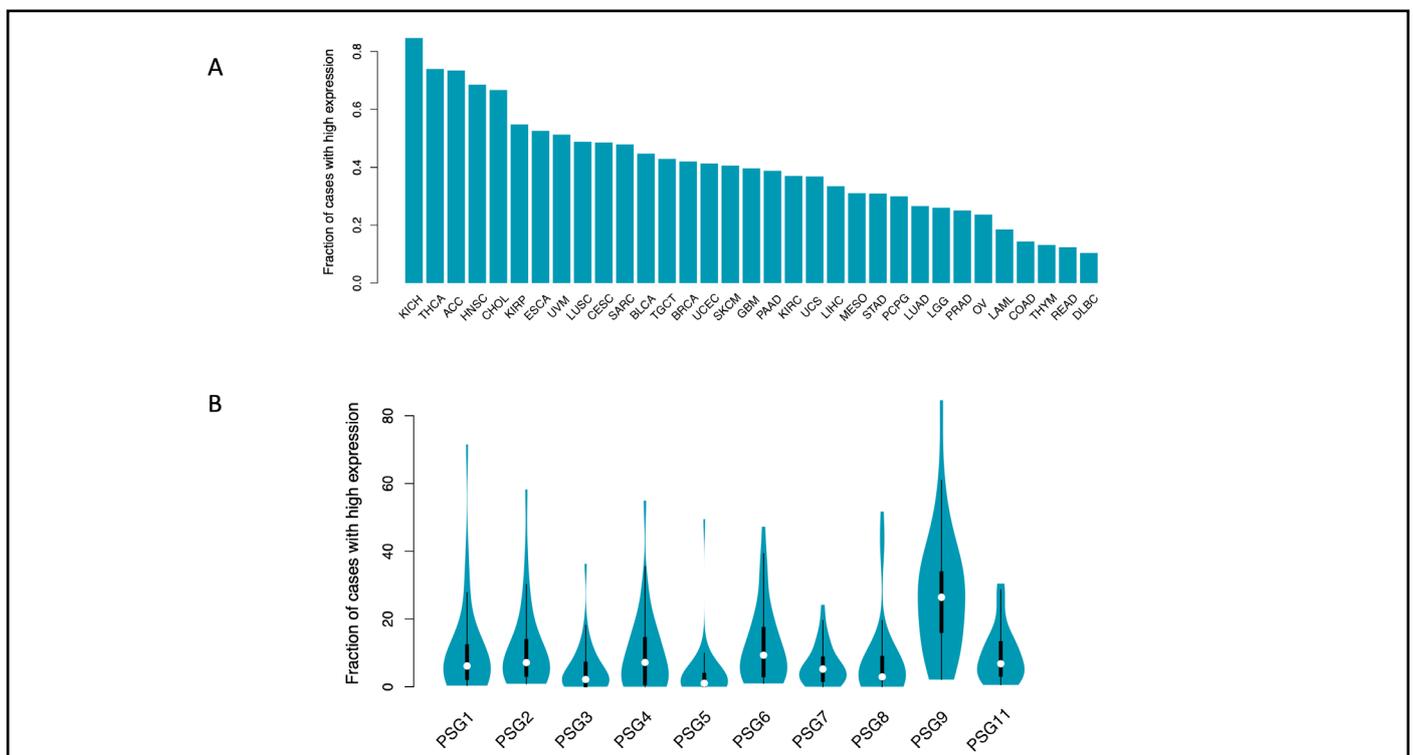
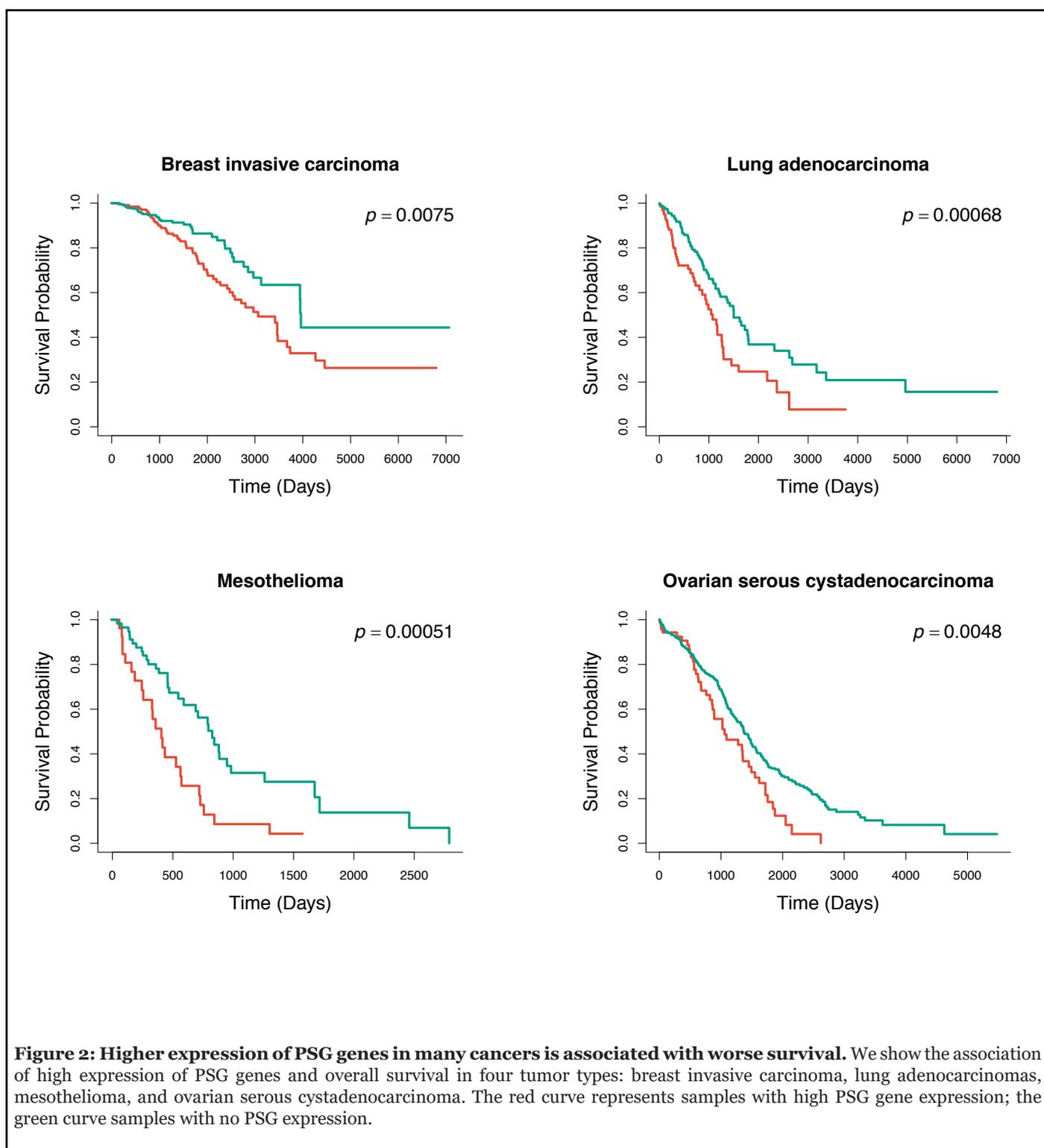
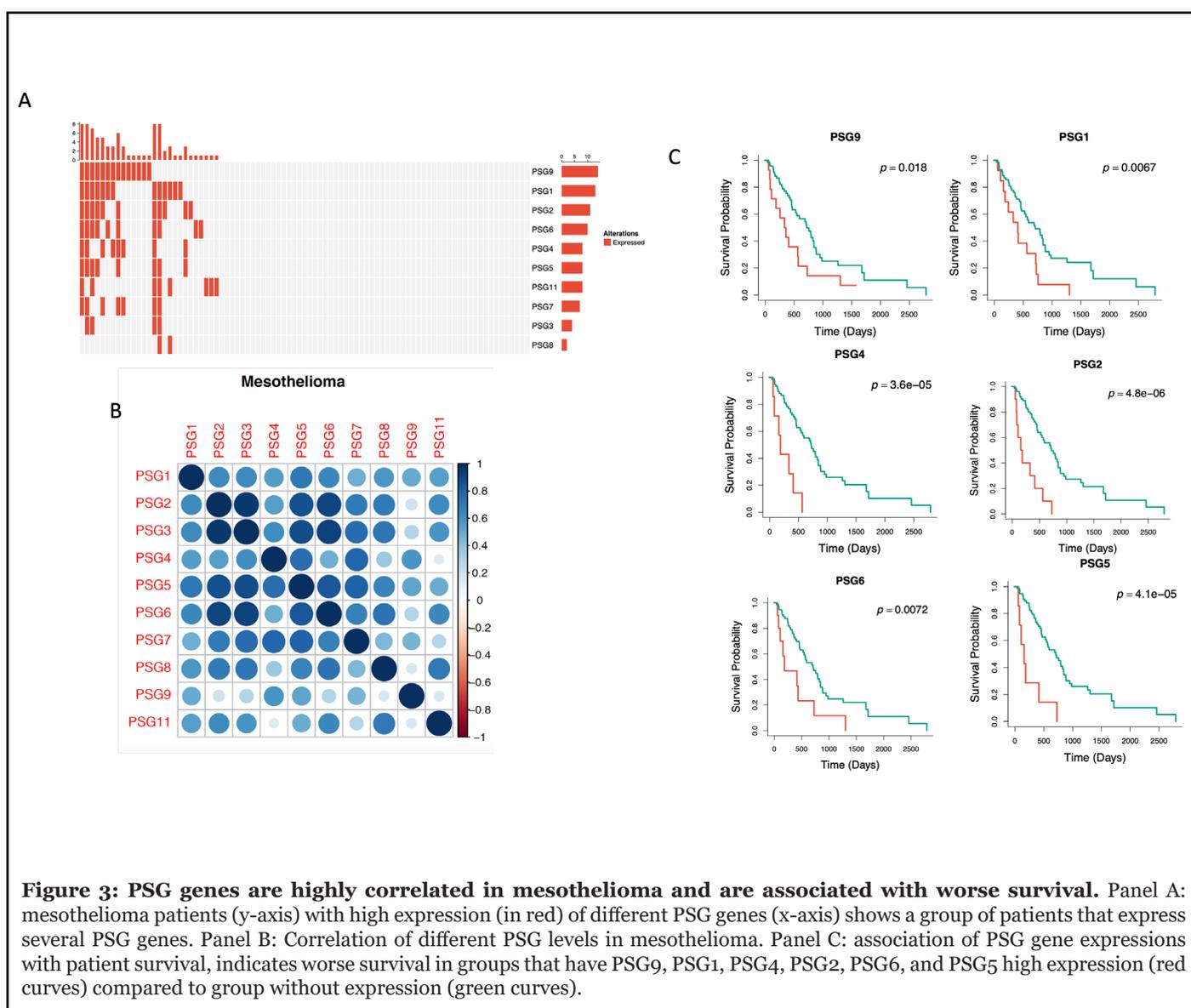


Figure 1: PSG genes are highly expressed across many tumors using 10,000 tumor data from The Cancer Tumor Atlas (TCGA). Panel A: Overexpression of PSG genes was common across many tumors types, reaching more than 60% of kidney chromophobe tumors, thyroid carcinomas, adrenocortical carcinomas, head and neck squamous cell carcinomas, and cholangiocarcinomas. Panel B: Fraction of tumors expressing PSG genes in TCGA shows different level of expression of these genes across tumor types.



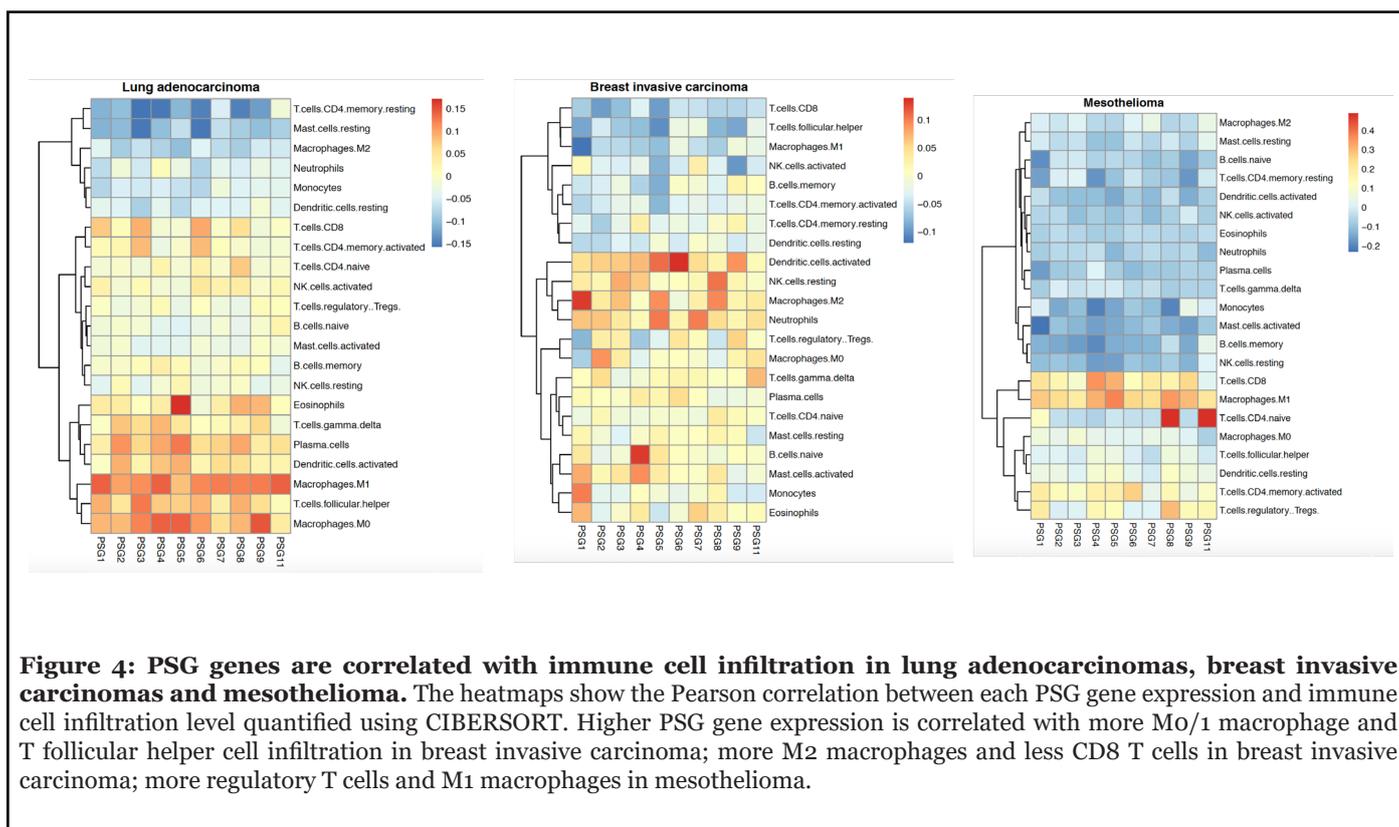
We next analyzed the relation between high expression of these genes and prognosis (overall survival). We compared the tumors expressing at least one of the PSG genes with the ones that do not significantly express any of these genes. We observed that in four tumor types: breast invasive carcinoma ($p=0.0075$), lung adenocarcinomas

($p=0.00068$), mesothelioma ($p=0.00051$), and ovarian serous cystadenocarcinoma ($p=0.0048$), patients with higher levels of PSGs present had a worse outcome (logrank test) (Figure 2). These results indicate that PSG upregulation is associated with clinical characteristics.



As an example, we explored in detail one of these tumors: mesothelioma. The TCGA mesothelioma collection includes 87 samples. When comparing the expression of different PSG genes in these tumors, we observed that 31% of these patients expressed at least one of the PSG genes, and that some patients expressed several (Figure 3A). This indicates that co-regulation of PSG genes is associated with a subset of patients. This observation was reinforced when observing the high correlation between the expression of these genes (Figure 3B). The association of overexpression of each of the genes with poor overall survival indicates that all these genes are associated with worse prognosis (Figure 3C). For instance, mesotheliomas highly expressing PSG-2 showed a median survival of the patient of 171 days vs the rest of mesotheliomas with 466 days. These results indicate that mesotheliomas expressing PSG genes constitute a worse prognosis subset.

Using CIBERSORT [19], we explored the association between PSG genes and different immune cell infiltrates. In Figure 4, we show the results for lung adenocarcinomas, breast invasive carcinomas, and mesothelioma. The results show a tumor specific association. In lung adenocarcinoma, the expression of PSG genes is significantly positively correlated with the level of Mo/1 macrophages (these are the target cells for PSGs that then secrete TGF-beta and IL-10 to produce suppressor T-regs) and T follicular helper cell infiltration, while negatively correlated with CD4 memory T cell and mast cells. In breast invasive carcinoma, higher PSG gene expression is associated with more M2 macrophages and fewer CD8 T cells. In mesothelioma, we found that the samples with higher PSG gene expression have more regulatory T cells and M1 macrophages. Clearly, the responses to the secretion of PSGs in the surrounding cellular matrix may differ with the tissue type of the tumor.



Discussion

The immune system in humans is sexually dimorphic. Many autoimmune diseases occur in females much more commonly than males, with a female to male ratio of 16/1 to 1.5/1 [6]. During pregnancy many of the symptoms of these autoimmune diseases are moderated, suggesting some similarities in the excesses observed with autoimmune diseases and their reduced activity during pregnancy. Females commonly have better outcomes with infections and sepsis than males do [6]. Females have a lower incidence of cancers over a lifetime than males, even though females live longer than males on average [6]. Males with cancers have better outcomes than females treated with checkpoint therapy [18]. A meta-analysis of 20 clinical trials with more than 11,000 patients treated with three different drugs (CTLA-4, PD-1, and PD-L1) employed with melanomas, renal cell carcinomas, urethral cancers, head and neck cancers and lung cancers was assembled. The endpoint of all these studies was the risk of death. The hazard ratio for women on checkpoint therapy compared to women not taking this therapy was 0.86. The hazard ratio of men taking this checkpoint therapy compared to women not taking these drugs was 0.72. The hazard ratio of males that took checkpoint therapy compared to men not taking this therapy had an even greater reduced risk of death than women taking checkpoint therapy [18]. These differences surely have genetic, hormonal, and even environmental effects upon these dimorphic properties. The requirement

to protect the fetus from the mother's immune system in viviparous species could well be related to the reasons for sexual dimorphisms that make the female immune system differ from the male immune system. In this way the sexual dimorphisms observed in cancer incidence and with the immune system and with checkpoint therapies could have some common causes.

The PSG genes are localized in a contiguous grouping on human chromosome 19.1-19.3. They have their evolutionary origins from a gene amplification followed by mutational diversity from an ancestor carcino-embryonic antigen- CAM gene (CEACAM), which is also located in this same chromosomal region [20]. Based upon the existing evidence, the pregnancy specific glycoproteins are good candidates to play an important role in reducing the CD-8 T-cell killing in both the fetus and in a variety of tumors in humans (Figure 1), [12-17]. A surprisingly large number of human tumors express reasonably high levels of PSG m-RNAs. The frequency of PSG gene expression in tumors depends upon the tissue type, but is very high (65-80% of the tumors) in renal clear cell carcinoma, thyroid carcinomas, adrenal cortico-carcinomas, head and neck squamous cell carcinomas and cholangial carcinomas. The frequency is moderately high (50-60%) in renal papillary carcinomas, esophageal carcinomas, uveal melanomas, non-small cell lung squamous carcinomas, cervical squamous cell carcinomas, and sarcomas. Eighteen other tissue types have frequencies of PSG expression between

20-50% (Figure 1). Although the m-RNA expression of most of the PSG genes in tumors is coordinately regulated, significant differences exist in tumor tissues, with PSG-9 being at the highest levels followed by PSGs 1, 2, and 6. It will be important to see if this translates to PSG protein levels in tumors, and whether or not one or more than one PSG is needed to determine their contribution to checkpoint therapies. Rather clearly, the Kaplan Meyer plots, at least for mesotheliomas, demonstrate that several PSGs (1, 2, 4, 5, 6, and 9) can contribute to the poor overall survival of the patient (Figure 3). The Kaplan Meyer plots for many tissue types (breast, lung, mesothelioma, serous ovarian, Figure 2) and uterus and colon [17] have suggested that the PSGs are playing a role in a large number of human cancers, contributing to poor overall survival. With all of the clinical trials ongoing in humans to search for immune tolerating factors in cancers, it is a bit surprising that the PSGs have been largely overlooked.

At least one of the mechanisms for the functions of PSGs is that they bind to a receptor on monocytes and macrophages and this triggers the secretion of preformed IL-10, TGF beta-1, and IL-6, which is packaged in vesicles that are then secreted from the monocyte [14,15]. IL-10 and TGF-beta -1 act upon CD-4 T-cells to make Fox P-3 + T-cell (regs) which act to suppress the CD-8 T-cell killing of cells presenting foreign antigens in HLA-class 1 receptors. The complexes formed between CD-8 T-cell receptor and the peptide antigen in the class 1 receptor is the place where CTLA-4 and PD-1 and PD-1 ligand act to turn off the killing of the tumor cell [5]. CD-4 and CD-8 T-cells (TILs) infiltrating into tumors are an indication of an immune response. Figure 4 attempts to examine the cell types that are infiltrating the tumors by employing the RNA sequencing data to identify cell types in and around the tumors when the tumors are producing PSGs. In several different tumor types, macrophages are present around the tumorous tissues. In lung and mesotheliomas there is an indication of some CD-8 T-cells, whereas in breast, CD-8 T-cells are not present. Clearly, tissue type appears to influence the patient's cellular response to the tumor. Only testing the PSGs with antibodies against them or their receptors on monocytes, will determine if tumor killing occurs and cellular infiltration differs for PSG positive tumors. The results presented here ought to encourage attempts to test whether antibodies directed against the PSGs will permit CD-8 T-cell killing of some of these tumor types.

Methods

RNA-seq data preparation

We downloaded normalized gene expression quantification data of TCGA samples from <https://portal.gdc.cancer.gov/> using TCGA-assembler [21]. For each

PSG gene, we applied a cutoff to determine whether it is expressed in one sample or not. The cutoff is defined as the 95% quantile of the expression level of each PSG gene in normal tissue from GTEx <https://www.gtexportal.org/>.

Survival analysis

TCGA clinical data was downloaded using TCGA-assembler [21] and then matched with RNA-seq data via the patient barcode provided by TCGA. For each study, the association of PSG genes with the survival outcomes was assessed via Cox proportional hazards regression using the 'coxph' function of the R 'survival' package. P value was obtained using the log-rank test.

Tumor immune cell infiltration analysis

CIBERSORT, a machine learning to estimate the abundances of member cell types in a mixed cell population, was applied to all RNA-seq data from TCGA [19]. For each data set, estimated mRNA fractions of each leukocyte subset were related to PSG gene expression using Pearson correlation. Then we used heatmap to visualize the result.

Statistical analysis

All the statistical analyses were conducted using R-3.6.3.

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