Trends in Immunology

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Opinion

Cancer germline antigens and tumor-agnostic CD8$^+$ T cell evasion

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Cancer germline antigens (CGAs) are expressed in immune-privileged germline tissues, while epigenetically silenced in somatic tissues. CGAs become re-expressed in tumors and can promote oncogenesis. Tumors prominently exploit mechanisms similar to those in germline tissues to shield from immunosurveillance. We hypothesize that CGAs contribute towards tumor escape from immune effector CD8$^+$ T cells. For illustrative purposes, we assessed the co-presence or -absence of CGAs with these cells in multiple tumor types. Considering a broad array of CD8$^+$ T cell evasive mechanisms, we exemplify the co-occurrence of gene transcripts of eight CGAs with those of adhesion molecules, endothelial cells, and/or the Wnt pathway. We present a novel concept of CGAs and their association with CD8$^+$ T cell evasion, which may be relevant for future immunotherapeutic interventions.

Highlights

Interrogation of the transcriptomics of multiple tumors demonstrates that example cancer germline antigens (CGAs) significantly correlate either with the presence or absence of intratumoral CD8$^+$ T cells in a pan-cancer manner.

Example CGAs co-occur with distinct perturbations of the presence, recognition, and/or activation of CD8$^+$ T cells, which may have been co-opted from immune-privileged noncancerous sites where CGAs are normally expressed.

Genes categorized into CD8$^+$ T cell evasive mechanisms, and correlated with genes identifying either adhesion molecules, endothelial cells, or the Wnt pathway, are frequently associated with exemplified CGAs, supporting the hypothesis that CGAs may be specifically related to cancer immunity.

We posit that by identifying the relationships between CGAs and CD8$^+$ T cell evasion, when further experimentally validated, it may be possible to provide a rationale for targeting CGAs, presumably to either remove tumor cells and/or render the tumor immune microenvironment more sensitized to infiltration by higher numbers of effector CD8$^+$ T cells.

Human CGAs

Human **CGAs** (see Glossary) comprise 276 proteins that are expressed in germline tissues, such as the testis, ovary, and placenta, and can be split according to their chromosomal locations, being either X- (n = 128) or non-X-linked (n = 148) [1,2]. In general, X-linked CGAs are expressed in the early phases of gametogenesis (the production process of reproductive cells), while the non-X-linked CGAs are expressed in the later stages of gametogenesis [1]. Within this class of proteins, several families have been identified that share homology, such as the MAGE, GAGE, SSX, and XAGE families [2]. Of note, identification of new CGAs with typical germline expression is still ongoing (CT database contains detailed and current information on CGAs)[i] [2].

CGAs become frequently re-expressed in tumors, where their expression has often been associated with poor clinical outcomes [2,3]. For instance, HORMAD1 and MAGEA3 expression has been linked to poor clinical outcomes in lung and prostate carcinoma, respectively [4,5]. Expression in human tumors is often a consequence of epigenetic changes, such as promoter demethylation and histone methylation or deacetylation, which are frequent events during gametogenesis [6]. In addition, the activation of transcription factors and signaling pathways has also been associated with CGA re-expression in human tumors, with examples being the E26 transformation-specific (ETS) [7] or CCCTC-binding factor like (CTCFL) (the latter being a CGA itself), as well as SP-1, p53, and cyclic AMP pathways [8–12].

It is well recognized that many CGAs promote oncogenic processes, such as cellular proliferation, genomic instability, invasion, and metastasis (for a timely overview, see [13]). In this opinion, we explore a new hypothesis regarding CGA biology, namely, that CGAs act against anticancer immunity, particularly that executed by CD8$^+$ T cells. We describe CD8$^+$ T cell evasion as being present in human immune-privileged healthy, as well as tumor, tissues. We provide illustrative examples of CGAs that associate with the presence or absence of CD8$^+$ T cells, as well as

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defined CD8$^+$ T cell evasive mechanisms in a **pan-cancer** manner. Finally, we discuss these examples in the context of experimental validation and assess their potential impact for future therapeutic interventions.

CGAs and CD8$^+$ T cell evasion: the first evidence

Germline tissues have several mechanisms at their disposal to overcome immunosurveillance [14]. First, reproductive tissue structures harbor barriers that prevent influx of immune cells. These barriers are either formed by tight junctions between Sertoli cells (the somatic cells of the testis that facilitate spermatogenesis) or the zona pellucida (the extracellular matrix of the ovary) [15,16]. Second, these tissues have T cell-suppressive environments that likely create immune privilege. For instance, murine Sertoli cells secrete molecules that suppress the proliferation and survival of immune cells, such as transforming growth factor β (TGF-β), activin A, FAS ligand, and granzyme B [17]. In another example, Sertoli, mononuclear, and endothelial cells in rats express Ido1 [18], which is required to prevent maternal CD8$^+$ T cell responses against the fetus [19]. Furthermore, Sertoli cells and spermatocytes highly express PD-L1 and the former cell type barely expresses MHC molecules [17,20]. These examples suggest that germline tissues have mechanisms at play that suppress CD8$^+$ T cells and that can be co-opted by tumors, as discussed later. We postulate that CGAs represent a modus operandi by which tumors hijack some of these CD8$^+$ T cell evasive mechanisms.

CD8$^+$ T cell evasion can generally be split into three main **T cell evasion categories**, namely, perturbations of influx and migration by CD8$^+$ T cells, antigen recognition by CD8$^+$ T cells, or CD8$^+$ T cell functions [21]. We have used these as a guide to explore the aforementioned hypothesis. The ability of human CD8$^+$ T cells to infiltrate a tumor can be limited by a lowered production of chemoattractants, such as CXCL9 and CXCL10 [22]. Next to that, downregulation of adhesion molecules, such as *ICAM1* on lymphocytes, also limits lymphocyte tumor infiltration [23]. Moreover, the migration of T cells can also be restricted by rigid and dense extracellular matrices [24]. Also, antigen recognition by CD8$^+$ T cells can be compromised by mutations and/or downregulated expression of molecules of the **antigen processing and presentation (APP)** pathway [25]. Lastly, CD8$^+$ T cell functions can be hindered by the expression of oncogenes [26,27], an altered energy metabolism [28], and/or the presence of **immune suppressor cells**, such as regulatory T cells (Tregs), tumor-associated macrophages, or myeloid-derived suppressor cells. T cell effector functions can also be modulated by the altered expression of co-inhibitory immune checkpoint molecules such as programmed cell death protein 1 (PD-1) and cytotoxic T lymphocyte antigen 4 (CTLA-4), or other **co-stimulatory receptors or ligands** such as CD28 and inducible T cell co-stimulator (ICOS) [29,30]. Detailed examples of such evasive mechanisms are provided later.

Even though avoiding immune destruction is an emerging and recognized hallmark of tumors, the proposed relationship between CGA and immune evasion is largely unknown. Certain reports hint at a role for CGAs in CD8$^+$ T cell evasion. One study showed that MAGE-A-positive human melanoma tumors were resistant to CTLA-4 blockade, possibly through dysregulation of autophagy [31]. Also, expression of type 2 MAGEs has been associated with a lack of immune cells, as evidenced by transcriptomics in human low-grade glioma tumors [32]. Furthermore, SEMG1 and SEMG2 overexpression has been shown to increase glycolysis in different tumor cell lines [33], which can limit the cytolytic capacities of T cells [28]. Building on these early suggestions, we sought to elucidate the relationship between CGAs and T cell evasion by analyzing publicly available databases and interpreting potential contributions in light of current knowledge.

CGAs and CD8$^+$ T cell evasion: a systematic search

To test our hypothesis and provide illustrative examples, we searched for correlates between CGAs and CD8$^+$ T cell evasion via a stepwise interrogation of the TCGA gene expression

Glossary

Antigen processing and presentation (APP): cellular machinery that enables surface expression of peptide–MHC complexes, which depends on cleavage of proteins into smaller fragments in either the cytosol or endosomes, loading them onto MHC molecules from the endoplasmic reticulum, and cycling the peptide–MHC complexes to the plasma membrane.
Cancer germline antigens (CGAs): class of 276 proteins, expression of which is restricted to non-adult gonadal tissues; they are strictly epigenetically regulated, can be re-induced in tumors, and generally are not in healthy adult tissues. These proteins have variable functions in biology, particularly related to the migration and invasion of gonadal cells.
Co-inhibitory receptors/ligands: (or immune checkpoints); receptors and ligands that, upon ligation, result in the deactivation of immune cells. Examples include programmed cell death protein 1 (PD-1) and its ligands 1 and 2 (PD-L1/2) and cytotoxic T lymphocyte antigen 4 (CTLA-4) and its ligands CD80 and CD86.
Co-stimulatory receptors or ligands: receptors and ligands that, upon ligation, result in an activation of immune cells. Examples include CD28 and its ligands CD80, CD86; and inducible T cell co-stimulator (ICOS) and its ligand (ICOSLG).
IFN type I: secreted cytokines with protective effects against viral infections and immunomodulatory effects towards innate immune cells, thereby enabling adaptive immune responses. IFN type I comprises IFNα, IFNβ, IFNω, ε, κ, as well as various pseudotypes, which are generally produced by immune cells and resident stromal cells.
IFN type II: secreted cytokines with protective effects against viral infections and immunomodulatory effects towards innate immune cells, thereby enabling adaptive immune responses. IFN type II comprises IFNγ, which is generally produced by activated T cells.
Immune effector cells: differentiated immune cells that can mediate and/or support a response against infected or aberrant (i.e., tumor cells). Examples of cells include cytotoxic CD8$^+$ T cells, nonregulatory CD4$^+$ T cells, dendritic cells (DCs).
Immune suppressor cells: can inhibit the functions of, for example, CD8$^+$

database of prevalent tumor types, which includes tumors of the bladder, breast, head and neck, lung, pancreas, prostate, and skin[ii]. The expression of all reported 276 CGAs was assessed for their correlation with the abundance of CD8$^+$ T cells in a total of 3631 tumor samples. When such a relationship was evident in most tumor types assessed, the expression of CGAs was further subjected to correlation analyses for 21 detailed subcategories of CD8$^+$ T cell evasion (as part of three main categories), which enabled assessing the co-occurrence of selected CGAs with defined mechanisms of CD8$^+$ T cell evasion. Lastly, the findings regarding example CGAs were linked to published reports to put our hypothesis into perspective.

Subset of CGAs that correlate with an abundance of intratumoral CD8$^+$ T cells

In the first step, gene expression of CGAs was correlated with a score of tumor infiltrating lymphocytes (TIL score) that provided a surrogate measure of CD8$^+$ T cell abundance within the tumor (illustrated in Figure 1A). This score showed high concordance with numbers of CD8$^+$ TILs and TCR-Vβ reads, the latter providing insight on the breadth and diversity of TCR repertoires and T cell responses [34]. Out of 276 CGAs, 138 CGAs were expressed in at least a single tumor type, 15 of which correlated with TIL scores in a pan-cancer manner (Figure 1). Six CGAs positively correlated with the TIL score, namely, *ACRBP*, *DDX43*, *GPAT2*, *IL13RA2*, *PIWIL1*, and *TMEM108*, whereas nine CGAs negatively correlated with the TIL score, namely, *CCDC110*, *GPATCH2*, *IGF2BP3*, *IGSF11*, *JARID1B*, *ODF2*, *TSGA10*, *TSSK6*, and *ZNF165* (Figure 1B). Of

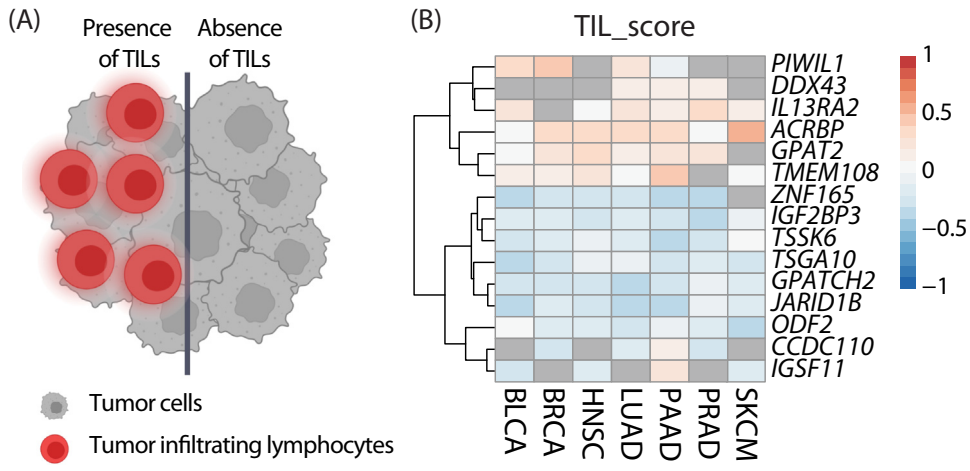

Figure 1. The gene expression of example cancer germline antigens (CGAs) correlates with tumor infiltrating lymphocyte (TIL) abundance in multiple human tumor types. (A) Cartoon of a tumor, characterized by either the presence or absence of TILs. (B) For illustrative purposes, a correlation heat map derived from TCGA gene expression datasets[ii] shows 15 examples of CGAs (out of 276; for complete list, see Table S1 in the supplemental information online) that were significantly correlated with a TIL score in a pan-cancer manner. Gene expression data of a total of 3631 human tumors of the skin (SKCM), head and neck (HNSC), bladder (BLCA), prostate (PRAD), breast (BRCA), lung (LUAD), and pancreas (PAAD) were extracted from the TCGA database[ii], standardized for transcript per million (TPM) expression level, and normalized according to gene lengths corrected trimmed mean of M-values (geTMM) [88]. TIL scores were calculated according to 109-gene signatures that have been previously reported [34]. Gene expression for 276 CGAs correlated with TIL scores when expressed in ≥3 tumor types (out of 7); for a given tumor type, the threshold for expression was reached when ≥25% of the samples had TPM values >1. Spearman's rank correlations between CGAs and TIL scores were considered pan-cancer when statistical significance was reached in at least two-thirds of the tumor types in which the particular CGA was expressed (i.e., two out of three, three out of four tumor types etc.); for a given tumor type, statistical significance for this correlation was reached when $r_s > 0.1$ or $r_s < -0.1$ and $P < 0.05$. Colors indicate the direction (red: positive; blue: negative) and extent of the correlations (low intensity: low; high intensity: high); grey indicates the absent expression of CGA in that tumor type (which excludes further assessment of correlations). On the left-hand side, a dendrogram is shown for illustrative purposes, displaying the evolutionary relationships among these 15 CGAs. This figure was created using BioRender (https://biorender.com/).

T cells. Examples include regulatory CD4$^+$ T cells (Tregs), tumor-associated macrophages (TAMs), and myeloid-derived suppressor cells (MDSCs).

Metabolic checkpoints: processes regulating the translation of a cell's energy status into an adaptive cellular response; can range from proliferation, senescence, to differentiation into immunomodulatory metabolites. Examples include adenosine and its energy-containing derivatives adenosine mono-, di-, and triphosphates; β-oxidation (linked to catabolism of fatty acids in the mitochondrion); electron transport chain; and mitochondrial activation (enhancing ATP levels in this organelle).

Oncogenic pathways: intracellular activation cascades linked to classical oncogenes, which, besides their contribution to cell growth, are recognized for their contribution to CD8$^+$ T cell evasion. Examples of oncogenic pathways with immune effects include mammalian target of rapamycin (mTOR), transforming growth factor β (TGF-β), vascular endothelial growth factor (VEGF), and wingless-related integration site (Wnt).

Pan-cancer: group of two or more tumor types. Examples of tumor types included in the assessment for pan-cancer expression of CGA or CD8$^+$ T cell evasion-related genes in this opinion article include bladder urothelial carcinoma (BLCA), breast invasive carcinoma (BRCA), head and neck squamous cell carcinoma (HNSC), lung adenocarcinoma (LUAD), pancreatic adenocarcinoma (PAAD), prostate adenocarcinoma (PRAD), and skin cutaneous melanoma (SKCM).

T cell evasion categories: perturbance of the presence, recognition, and/or activation of immune effector cells, thereby providing escape from an immune response, specifically, a CD8$^+$ T cell response. This evasion may confer tumors a 'license to survive' and is often adopted from the same escape paths that have established immune privilege. The three main categories of CD8$^+$ T cell evasion (i.e., limiting the presence, recognition, and/or activation of immune effector cells) are further divided into 21 subcategories that represent more detailed paths providing escape from an immune response.

note, classical CGAs, such as members of the MAGE family, did not significantly correlate with the TIL score according to our pan-cancer analyses. This is intriguing because these were the first CGAs to be identified and are widely studied. However, we cannot rule out that members of the aforementioned CGA families might be associated with CD8$^+$ T cell presence in either single or a limited number of tumor types.

Individual CGAs that preferentially associate with distinct mechanisms of CD8$^+$ T cell evasion

In the second step, of the 15 example CGAs from the previous step, gene expression was correlated with 21 gene sets that captured different subcategories of CD8$^+$ T cell evasive mechanisms (Figure 2A, Key figure). Of the three main categories, T cell influx and migration was subdivided into (i) stromal barrier, including endothelial cells and fibroblasts, and (ii) T cell recruitment, including adhesion molecules, chemoattractants, and immune effectors cells. Antigen recognition (i.e., APP) was subdivided into (i) **IFN type I**, (ii) **IFN type II** pathways, (iii) MHC and MHC accessory molecules, and (iv) other genes involved in APP. T cell function was subdivided into (i) **oncogenic pathways**, including mTOR, TGF-β, VEGF, and Wnt pathways; (ii) **metabolic checkpoints**, including adenosine, glycolysis, β-oxidation, electron

Key figure

The gene expression of example cancer germline antigens (CGAs) correlates with CD8$^+$ T cell evasive mechanisms in multiple human tumor types

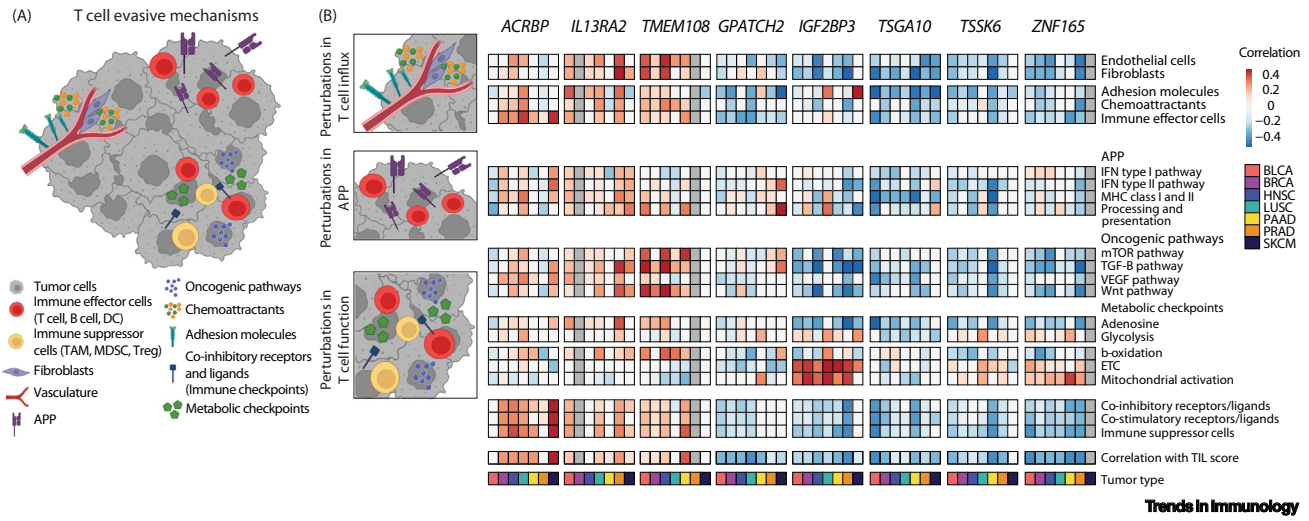

Figure 2. (A) Cartoon of three main categories of CD8$^+$ T cell evasion as previously described [21]; gene expression perturbations were considered for the following parameters: influx, antigen recognition, and function of CD8$^+$ T cells. (B) An illustrative correlation heat map showing eight examples of CGAs that were significantly correlated with expression scores of ≥1 subcategories of CD8$^+$ T cell evasive gene sets (out of 21; complete list in right-hand side of figure). All these gene sets consist of a total of 1090 genes according to [21] and represent different mechanisms in at least one of the three main categories for CD8$^+$ T cell evasion. Gene expression scores of each subcategory of CD8$^+$ T cell evasion for the different types of tumors were extracted from the TCGA database[ii] and processed as described in the legend to Figure 1B. Gene expression scores of the 15 CGAs from Figure 1B were correlated with genes from each gene set using normalized averages of all members per set [34]. Spearman's rank correlations between CGAs and CD8$^+$ T evasive subcategories were considered statistically significant when $P < 0.05$. Color coding of heat map is as described in the legend to Figure 1B, and color coding for tumor types is listed in Figure 2B. Bottom row: correlations between CGAs and tumor infiltrating lymphocyte (TIL) scores (from Figure 1B) are displayed as a reference. This figure was created using BioRender (https://biorender.com/). Abbreviations: APP, antigen processing and presentation; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; DC, dendritic cell; ETC, electron transport chain; HNSC, head and neck squamous cell carcinoma; LUSC, lung squamous cell carcinoma; MDSC, myeloid-derived suppressor cell; PAAD, pancreatic adenocarcinoma; PRAD, prostate adenocarcinoma; SKCM, skin cutaneous melanoma; TAM, tumor-associated macrophage.

transport chain (ETC), and mitochondrial activation; (iii) co-inhibitory (i.e., immune checkpoints) and co-stimulatory receptors/ligands; and (iv) immune suppressor cells. Sets of genes that represent each of these subcategories of $CD8^+$ T cell evasion have been previously reported [34]. In our example analysis, we observed that eight out of the above 15 CGAs were predominantly associated with one to five out of these 21 subcategories of $CD8^+$ T cell evasion (Figure 2B). We further discuss the exemplified CGAs and the possible implications of these correlations in the next section. The challenges of our example analysis, and the outcomes of this exploration, are highlighted in the last section.

CGAs and $CD8^+$ T cell evasion: defined examples

ACRBP, *IL13RA2*, and *TMEM108* positively correlate with TIL score

In this exercise, expression of the gene that encodes ACRBP positively associated with expression of genes that represent **immune effector cells** as well as co-stimulation, which could be expected since this CGA correlated positively with TIL score. Counterintuitively, *ACRBP* also positively associated with genes that represent immune suppressor cells or co-inhibition. We speculate that these latter positive associations are explained, at least in part, by an initial co-occurrence of *ACRBP* with an intratumoral T cell response, followed by a negative feedback loop. Such adaptive immune responses were reported by a seminal study in which human $CD8^+$ T cell-inflamed melanoma metastases showed higher expression of IDO, PD-L1, and Treg markers, compared with noninflamed melanoma metastases [35]. Various tumors with inflammatory phenotypes in which immune effector and suppressor cells coexist in equilibrium within the tumor microenvironment were recently reported and deeply characterized [36,37]. Along these lines, we postulate that the positive association that was detected between the expression of *ACRBP* and that of genes that represent immune suppressor cells or co-inhibition molecules may result from an adaptive response to keep antitumor $CD8^+$ T cells in check.

Expression of *IL13RA2* was positively associated with expression of genes that represent endothelial cells, adhesion molecules, and the TGF-β or Wnt pathways. Of note, *Il13ra2* was reported to enhance angiogenesis in xenograft melanoma tumors in mice [38], which is aligned with the association that was noted between *IL13RA2* expression and genes relevant for endothelial cells and adhesion molecules. Furthermore, IL13RA2 has been described to mediate IL-13-induced TGF-β1 production in human macrophages *in vitro*, and preventing *Il13ra2* expression in mouse models of colitis and lung fibrosis reduced the production of *Tgfb1* [39]. Indeed, TGF-β has been widely recognized as a suppressor of $CD8^+$ T cell proliferation and function [40,41]. For instance, TGF-β signaling has limited ovalbumin-specific T cell responses in thymoma mouse models in which genes encoding cytolytic and effector molecules (e.g., perforin, granzymes A and B, or IFN-γ) were silenced [42]. Building on these data, we speculate that the IL13Rα2-TGF-β axis might contribute to $CD8^+$ T cell evasion, although this remains to be rigorously assessed. From another angle, the Wnt pathway is also known for its adverse effects on T cell-mediated immunity. For example, active β-catenin signaling in autochthonous mouse melanoma models resulted in the absence of $CD3^+$ T cells in tumor areas [43]. In addition, *Il13ra2*-knockout mice showed reduced Wnt signaling compared with wild-type mice [44]. Taken together, our supposition that *IL13RA2* might be associated with $CD8^+$ T cell evasion extends these earlier reports, but certainly warrants further investigation into the putative involvement of specific signaling pathways.

Expression of *TMEM108* was positively associated with expression of genes relevant for endothelial cells, the Wnt pathway, or β-oxidation. Of note, Tmem108 was described as a transmembrane protein required for cognitive functions in a *Tmem108* loss-of-function mutant mouse model [45]. Also, Tmem108 has been suggested to regulate adult neurogenesis in mice through

the Wnt/β-catenin pathway [46], and a mutation in the gene encoding Tmem108 was reported to induce glucose intolerance and increased insulin resistance, suggestive of a disturbed metabolism in these mice [47]. One might speculate that these functions are consistent with the associations observed between this CGA gene and the Wnt pathway or β-oxidation, yet reports regarding the role of *TMEM108* in T cell immunity in general, or in endothelial cells specifically, are lacking.

GPATCH2, *IGF2BP3*, *TSGA10*, *TSSK6*, and *ZNF65* negatively correlate with TIL score

Expression of *GPATCH2* was negatively associated with expression of genes relevant for endothelial cells, adhesion molecules, or immune effector cells. Of note, the GPATCH2 protein interacts with the RNA-dependent ATPase DHX15, enhancing its activity in human breast cancer cells *in vitro* [48]. DHX15 is involved in activating the NF-κβ pathway in leukemic cell lines *in vitro* [49]. In contrast, GPATCH2 has also been suggested to inhibit the NF-κβ pathway in human 293T cells *in vitro* [50]. Of note, inhibition of NF-κβ has decreased the expression of vascular endothelial growth factor (*VEGF*) by human breast cancer cells *in vitro* and promoted the expression of *VCAM1* and *ICAM1* by human endothelial cells *in vitro* [51], thus facilitating transendothelial migration of immune effector cells. These examples have prompted the reasonable supposition that *GPATCH2* may be linked to NF-κβ signaling. Whether *GPATCH2* expression activates or inhibits NF-κβ signaling, and which immune effects are exactly promoted, seems to be context-dependent. Overall, we argue that these findings align with the negative association that was detected between *GPATCH2* expression and that of adhesion molecules, endothelial cells, and immune effector cells.

IGF2BP3 was the only CGA whose expression was uniquely and positively associated with the expression of genes representing metabolic checkpoints, particularly those related to mitochondrial activation or ETC. Of note, dysregulated metabolism can limit human and murine $CD8^+$ T cell activation and function [28]. Noteworthy, the expression of genes for mitochondrial activation and ETC negatively correlated with genes in other subcategories, such as adhesion molecules, immune suppressor cells, and co-inhibitory molecules; although the significance of these findings remains obscure, the noted associations may aid in understanding how changes in metabolic checkpoints can result in limited $CD8^+$ T cell immunity. According to earlier publications, even though *IGF2BP3* has not been directly associated with metabolism, this CGA has been reported to act as a binding partner for the circular RNA circCDKN2B-AS1 in human cervical cancer cell lines, which stabilizes the mRNA of *HEXOKINASE 2* (*HK2*), an enzyme that is involved in the aerobic glycolysis pathway [52]. Of note, *IGF2BP3* overexpression has resulted in increased expression of NF-κβ pathway genes in human renal cancer cells *in vitro*, suggesting that this CGA can also act on the NF-κβ pathway [53]. Indeed, knockdown of the NF-κβ pathway has led to reduced expression of *HK2* in human sarcoma cells *in vitro*, thereby promoting aerobic glycolysis [54]. Earlier reports have already demonstrated that glycolysis contributes to $CD8^+$ T cell evasion. For example, glycolysis-related genes have exhibited increased expression in melanoma patients who did not respond to adoptive T cell therapy and, furthermore, tumor cells derived from these patients demonstrated increased glycolytic activity *in vitro* when compared with tumors of responding patients [28]. According to our analysis, *IGF2BP3* positively associated with genes for glycolysis, albeit to a lesser extent than for mitochondrial activation or ETC (Figure 2). Thus, *IGF2BP3* may act as a glycolysis regulator via *HK2* and NF-κβ signaling, presumably impacting metabolic events within the tumor microenvironment; if true, this might contribute to explain the co-occurrence of *IGF2BP3* with transcripts relevant for metabolic checkpoint genes.

TSGA10 expression was negatively associated with the expression of genes that represent fibroblasts, adhesion molecules, as well as MHC molecules. TSGA10 is considered to negatively

regulate the HIF-1α pathway [55]. In fact, TSGA10 was found to interact with HIF-1α in human HeLa cells, thereby preventing its binding to coactivators and downstream induction of the expression of target genes such as *VEGF* [55]. It is noteworthy that expression of the gene encoding Hif-1α in myeloid cells was reported as essential for the presence of cancer-associated fibroblasts (CAFs) in intestinal tumor mouse models and HIF-1α promoted the transformation of human fibroblasts to cells with a CAF phenotype [56,57]. Since *TSGA10* may negatively regulate HIF-1α, this might align with the negative association we observed between this CGA and genes identifying fibroblasts. In addition, TSGA10 is expressed by human antigen-presenting cells and interacts with vimentin, which is a component of the extracellular matrix produced by fibroblasts [58]. Of note, the absence of vimentin has been shown to reduce the expression of Icam-1 and Vcam-1 by mouse endothelial cells [59]. It is tempting to consider an indirect link between TSGAG10 and adhesion molecules via vimentin, whereby TSGA10 might provide a decoy binding partner for vimentin and negatively impact T cell movement across the endothelium, but this remains conjectural. Also, the negative association between *TSGA10* and MHC genes has not been observed for the other CGAs, which may suggest that *TSGA10* might be uniquely linked to limited APP, perhaps representing another angle of $CD8^+$ T cell evasion, although this remains to be tested.

Expression of *TSSK6* was negatively associated with the expression of genes that represent adhesion molecules. This CGA is reported to be involved in DNA condensation during chromatin remodeling and to interact with heat shock proteins, such as HSP90, in human 293T cells [60,61]. There has not been any report linking *TSSK6* to adhesion molecule genes. However, as discussed for *GPATCH2* or *TSGA10*, one might argue that a negative effect, either direct or indirect, of *TSSK6* on the expression of adhesion molecules, might result in poor intratumoral T cell infiltration, although this remains hypothetical; however, if true, this would align with the observed negative correlation between this CGA and TIL score.

Expression of *ZNF165* was negatively associated with the expression of genes identifying endothelial cells, fibroblasts, immune suppressor cells, as well as those involved in TGF-β and Wnt pathways. ZNF165 has been identified before as a regulator of the TGF-β pathway [3,62]. For example, in a triple negative breast cancer (TNBC) xenograft mouse model, ZNF165 formed a transcriptional complex that drove TGF-β signaling towards a more oncogenic program. Also, *ZNF165* suppressed the expression of TGF-β negative feedback regulators *SMURF2* and *SMAD7* in human TNBC cell lines *in vitro* [3,62]. In contrast to the TGF-β pathway, *ZNF165* expression has not been previously reported to associate with genes identifying fibroblasts, endothelial cells, immune suppressor cells, and/or the Wnt pathway. However, it is tempting to speculate that the TGF-β pathway might contribute to an immune-excluded or ignored tumor phenotype via its (in)direct actions on other subcategories of $CD8^+$ T cell evasion [63].

In sum, we have identified eight examples of CGAs that were predominantly associated with at least one subcategory of $CD8^+$ T cell evasion. Of the 21 subcategories, 13 subcategories showed a significant correlation with at least one CGA in multiple tumor types and were presumed as co-occurring evasive mechanisms with the respective CGA (Figure 2). To delineate which 'evasive' mechanisms (according to the categorization) occurred most frequently in multiple tumor types, we calculated enrichments for each mechanism relative to all occurring mechanisms. In case a single perturbed $CD8^+$ T cell evasion mechanism represented more than 12% of all perturbations, this equaled a more than 2.5-fold enrichment; the defined evasions in this exercise are depicted for the eight examples of CGAs (Figure 3A). Perturbations that were linked to adhesion molecules, endothelial cells, or the Wnt pathway co-occurred most frequently with these CGAs (Figure 3B). Given the large multitude of CGAs and $CD8^+$ T cell evasive pathways,

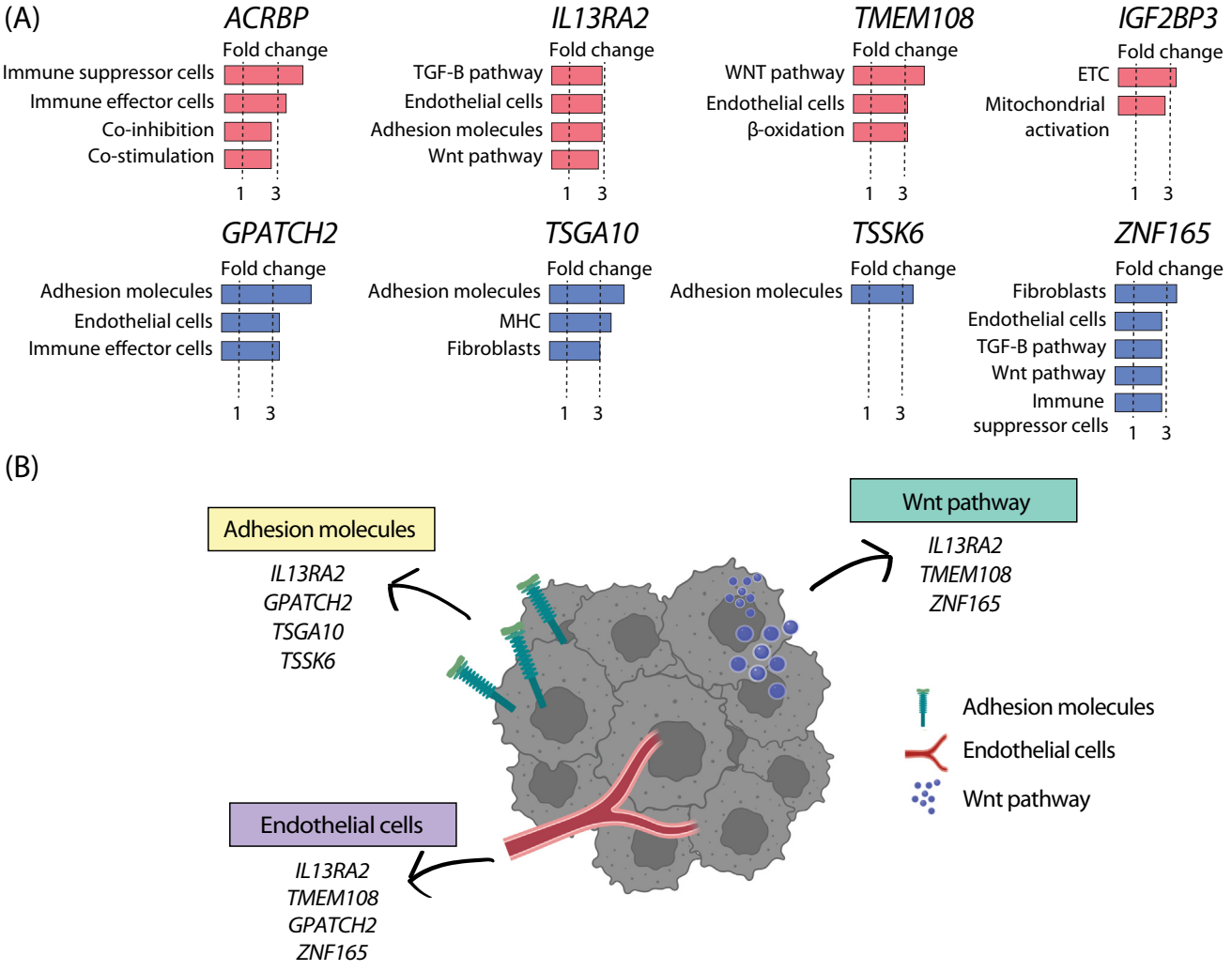

Figure 3. Example cancer germline antigens (CGAs) can associate with distinct CD8$^+$ T cell evasive mechanisms in multiple tumor types. (A) Illustrative graphs show eight examples of CGAs that demonstrated significant enrichments in calculated versus random co-occurrence of ≥1 distinct CD8$^+$ T cell evasive subcategory. To this end, significant correlations between CGAs and CD8$^+$ T cell evasive mechanisms from Figure 2B were expressed relative to the sum of correlations for all gene sets for all tumors and for a particular CGA. Relative Spearman's rank correlations between CGAs and CD8$^+$ T cell evasive gene sets were considered statistically significant when $r_s > 0.3$ or $r_s < -0.3$ and $P < 0.05$ in those tumor types where the CGA met the criteria for gene expression (similar to Figure 1B). For each of the eight exemplary CGAs, the CD8$^+$ T cell evasive subcategories are listed from high to low enrichment. Fold changes were expressed relative to a theoretical occurrence of 1 out of 21 (being 4.8%). Colors indicate the direction (red: positive; blue: negative) of enrichment of the perturbed CD8$^+$ T cell evasive mechanisms. (B) Cartoon of CD8$^+$ T cell evasive mechanisms (as represented by enrichments from A) that were perturbed for ≥three CGAs. This figure was created using BioRender (https://biorender.com/). Abbreviation: ETC, electron transport chain.

this outcome might be surprising (see Outstanding questions). To address our hypothesis, tumor-agnostic CD8$^+$ T cell evasion might rely on a limited number of CGAs, and/or a limited number of pathways, a possibility that warrants further investigation. A complete overview of the aforementioned eight CGAs and their correlations with CD8$^+$ T cell evasive mechanisms is shown in Table 1 (and Tables S1 and S2 in the supplemental information online), in which chromosomal locations, cellular compartments of expression, reported biology, as well as presumed functions in oncogenesis are listed.

Table 1. Human example CGAs and their proposed relationship with mechanisms of CD8$^+$ T cell evasion, biology, and oncogenesis

Example CGA[a]	T cell evasive parameter	Chromosome	Cellular compartment	Biology	Oncogenesis	Refs
ACRBP	Positively associated with: - TIL score - Immune suppressor cells - Immune effector cells - **Co-inhibitory receptors/ligands** Co-stimulatory receptors/ligands	12	Nucleus in acrosomal vesicle	- Forms acrosome - Maintains pro-acrosin as an enzymatically inactive zymogen for proper biogenesis of the acrosome	- Supports mitotic spindle dynamics in ovarian cancer cell lines and ovarian tumor explants - Negatively affects expression of apoptosis-regulated protein caspase-3 in hepatocellular carcinoma cell lines - Downregulated expression of ACRBP decreases expression of cell cycle-regulated protein cyclin E and migration/invasion regulated proteins MMP2 and MMP9 in hepatocellular carcinoma cell lines	[74–76]
IL13RA2	Positively associated with: - TIL score - Endothelial cells - TGF-β pathway - Wnt pathway - Adhesion molecules	X	Membrane/ extracellular region	- Acts in part as decoy receptor for IL-13 that lacks canonical JAK and STAT signaling - Activates several pathways (i.e., TGF-β1, ERK/AP-1, and SRC/PI3K/AKT/mTOR pathways) - Mediates Chi3l1-induced cellular responses	- Enhances angiogenesis in malignant melanoma *in vivo* - Induces invasion and metastasis in several tumor types *in vitro* and *in vivo* - Interacts with CHI3L1 protein on the plasma membranes of cancer cells, leading to the upregulated expression of matrix metalloproteinase genes and tumor metastasis	[38,39,44, 77–82]
TMEM108	Positively associated with: - TIL score - Endothelial cells - Wnt pathway β-oxidation	3	Endosome membrane/ axon	- Is a transmembrane protein required for proper cognitive functions	Not reported	[45]
GPATCH2	Negatively associated with: - TIL score - Adhesion molecules - Endothelial cells Immune effector cells	1	Nucleus	- Enhances ATPase activity of DHX15	- Enhances activity of DHX15, which in turn has a growth-promoting effect on mammalian cells. DHX15 can also either activate NF-kB pathway in leukemia cells *in vitro* or inhibit NF-kB pathway in human 293T cells *in vitro*.	[48–50]
IGF2BP3	Negatively associated with: - TIL score Positively associated with: - ETC Mitochondrial activation	7	Nucleolus	- Promotes cell adhesion and invadopodia formation - Is an RNA-binding factor that recruits target transcripts to cytoplasmic protein–RNA complexes	- Promotes oncogenic growth through regulation of mRNA stability and mRNA–microRNA interactions - Involved in cell adhesion and invadopodia formation in HeLa cells - Impacts on the expression and function of oncoproteins and tumor suppressor proteins	[83,84]
TSGA10	Negatively associated with: - TIL score - Adhesion molecules - Fibroblasts MHC molecules	2	Cytoplasm	- Involvement of C terminus in centriole assembly and function, especially in the sperm head–tail connection	- Induces disruption of the HIF-1α axis in HeLa cells - Inhibits tumor growth, angiogenesis, and metastasis in HeLa cells - Act as tumor suppressor gene in human esophageal squamous cell carcinoma	[55,85,86]
TSSK6	Negatively associated with: Adhesion molecules	19	Nucleus and cytoplasm	- Plays a role in DNA condensation during postmeiotic chromatin remodeling	- Interacts with heat shock proteins HSP90 and HSP70	[61]

(continued on next page)

Table 1. (continued)

Example CGA[a]	T cell evasive parameter	Chromosome	Cellular compartment	Biology	Oncogenesis	Refs
ZNF165	Negatively associated with: - TIL score - Endothelial cells - Fibroblasts - Wnt pathway - TGF-β pathway Immune suppressor cells	6	Nucleus	- Involved in transcriptional regulation	- Modulates transcription of TGF-β-dependent genes and thereby promotes growth and survival of triple negative breast cancer - Stimulates cell proliferation and antiapoptosis through NF-κβ, TGF-β, and PI3K pathways in HeLa cells	[3,62,87]

[a]Full gene names and gene identifiers are listed in Table S1 in the supplemental information online.

Challenges of this CGA analysis and future directions regarding 'CD8$^+$ T cell evasive' CGAs

With the earlier examples, we propose that CGAs are positively or negatively correlated with the abundance of TILs and may specifically hamper CD8$^+$ T cell influx and/or function in a pan-cancer manner, particularly through mechanisms related to adhesion molecules, endothelial cells, and/or the Wnt pathway. These data do not exclude that CGAs may behave differently from each other and/or according to different tumor types. In addition, the outcome of our hypothesis test does not exclude the possibility that aside from the three alluded mechanisms of 'evasion', other mechanisms of CD8$^+$ T cell evasion may be related to the eight example CGAs. Indeed, we observed co-occurrence of CGAs, albeit less frequent, with immune checkpoints (for *ACRBP*), metabolic checkpoints (*IGF2BP3*), the presence of immune suppressor cells (*ACRBP*, *ZNF165*), the TGF-β pathway (*IL13RA2*, *ZNF165*), the presence of fibroblasts (*TSGA10*, *ZNF165*), or the low expression of MHC molecules (*TSGA10*).

We appreciate that the presented analysis has many limitations and caveats. Notably, the observed correlations suggest co-occurrence but do not provide proof for a causal relationship between CGAs and the presence or usage of CD8$^+$ T cell evasive mechanisms. This notion is further exemplified by a marked positive correlation among the subcategories of T cell evasion, except for metabolic checkpoint genes (see previous section, *IGF2BPF3*). Also, within single categories of CD8$^+$ T cell evasion, certain genes may dominate, or not, an association that becomes 'diluted' when looking at the average gene expression for a complete set of genes. In addition, the context dependency of the tumor microenvironment is not taken into account here. For example, RNA sequencing data used in our example analyses are obtained from bulk tumors and do not pinpoint the exact cellular source of expressed CGAs or immune-related genes. Although the points raised in this opinion article regarding specific CGAs and CD8$^+$ T cell evasion are timely, they are also largely speculative. To begin validating these associations and mechanisms of action for example CGAs, we propose that tumor tissues be assessed for spatial organization of particular CGA proteins via immunostaining. For example, localized expression of CGA proteins in the marginal versus center area of the tumor, and the relationship with tumor CD8$^+$ T cell phenotypes (i.e., T cell-ignored, -excluded, or -inflamed), can be assessed. Such an assessment might aid to better understand the effects of CGAs in modulating T cell numbers, subsets, as well as functions. In addition, *in vitro* assays in which T cells are cocultured with 2D or 3D tumor cells/organoids that either overexpress or exhibit knocked-down/loss of expression of such CGAs may help to assess whether CGAs directly affect CD8$^+$ T cell numbers and functions. Furthermore, *in vivo* experiments with immunocompetent mouse models and the use of imaging techniques (e.g., two-photon intravital microscopy) may enable the study of indirect effects of CGAs on CD8$^+$ T cell numbers and functions; however, the use of

immunocompetent mouse models might also be challenging because many X-linked CGAs do not have murine orthologs.

Concluding remarks

CGAs have been considered relevant for immunotherapies due to their immunogenicity and ability to induce CD8$^+$ T cell responses in cancer patients [64–66]. In fact, certain CGAs, such as NY-ESO1, MAGEA1, MAGEA3, MAGEA4, and MAGEC2, have been or are being explored as putative vaccines or targets for adoptive T cell therapy in several cancers, such as melanoma, multiple myeloma, synovial sarcoma, and head and neck cancers [67–73]. Besides targeting CGAs via T cell receptor-engineered T cells to destruct tumor cells, future immunotherapies could also benefit from actionable targets to improve antitumor CD8$^+$ T cell immunity. Accordingly, we argue that CGAs may potentially be targeted via adoptive T cell therapy, drugs, antibodies, or nanobodies, presumably to impair CD8$^+$ T cell evasion (see Outstanding questions). If our hypothesis is proven to be correct, and given the pan-cancer expression of some CGAs, multiple cancer types might become sensitized to immunotherapies when using presumed future anti-CGA therapies in a rationalized and/or complementary manner.

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Declaration of interests

No interests are declared.

Supplemental information

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Resources

[i]www.cta.lncc.br/index.php

[ii]www.cancer.gov/tcga

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Outstanding questions

Why are the CGAs *ACRBP*, *DDX43*, *GPAT2*, *IL13RA2*, *PIWIL1*, and *TMEM108* positively correlated with the presence of intratumoral CD8$^+$ T cells, whereas the CGAs *CCDC110*, *GPATCH2*, *IGF2BP3*, *IGFS11*, *JARID1B*, *ODF2*, *TSGA10*, *TSSK6*, and *ZNF165* are inversely correlated with the presence of intratumoral CD8$^+$ T cells?

Why are only eight CGAs correlated to presumed CD8$^+$ T cell evasive mechanisms in a pan-cancer manner? Does this suggest that most CGAs might be correlated to CD8$^+$ T cell evasive mechanisms, if at all, in a single tumor type?

Why do some CGAs, such as *IL13RA2* and *TMEM108*, correlate with multiple presumed CD8$^+$ T cell evasive mechanisms, while other CGAs, such as *TSSK6*, only correlate with a single CD8$^+$ T cell evasive mechanism? In addition, why do some CGAs, such as *IL13RA2*, show a positive enrichment for gene sets representing adhesion molecules, while other CGAs, such as *TSSK6*, show a negative enrichment for gene sets representing adhesion molecules?

What are the environmental cues that affect the expression of CGAs and the associated CD8$^+$ T cell evasion mechanisms? Can we modulate these environmental cues and does this modulate CD8$^+$ T cell evasion?

What are the exact molecular mechanisms of action of the eight example CGAs towards CD8$^+$ T cell presumed evasive mechanisms, particularly in terms of perturbations linked to adhesion molecules, endothelial cells, or the Wnt pathway? Would assessment of *in situ* expression and spatial organization of CGA proteins, *in vitro* effects of CGA-expressing tumor cell lines on immune cells, as well as *in vivo* effects of CGA-positive tumors on immune cells in mice support further validation for this model and help identify putative mechanisms of action?

What is the impact of targeting the example CGAs for putative immunotherapeutic interventions? Would this render the tumor

microenvironment more susceptible to being targeted by the immune system?

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