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Co-inhibitory Blockade While Preserving Tolerance: Checkpoint Inhibitors for Glioblastoma

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Summary

The introduction of immunotherapy with checkpoint receptor blockade has changed the treatment of advanced cancers, at times inducing prolonged remission. Nevertheless, the success rate of the approach is variable across patients and different tumor types, and treatment is often accompanied by severe immune-related side effects, suggesting the importance of co-inhibitory pathway for both prevention of autoimmunity and failure of tumor rejection. A better understanding of how to uncouple anti-tumor activity from loss of self-tolerance is necessary in order to increase the therapeutic efficacy of checkpoint immunotherapy. In this review, we describe basic concepts of T cell exhaustion that occur in cancer, highlighting the role of co-inhibitory receptors in contributing to this process while preventing immunopathology. By providing an overview of the current therapeutic success and immune-related burden of secondary effects of checkpoint immunotherapy, we illustrate the “double-edged sword” related to interference with immune-regulatory pathways. Finally, since achieving tumor rejection while preserving self-tolerance is particularly important for the central nervous system, we analyze the case for checkpoint immunotherapy in glioblastoma, the most common adult brain tumor.

Keywords

T cell exhaustion; tolerance; coinhibitory receptor; glioblastoma

Introduction

Immunotherapy represents a breakthrough in the management of advanced cancers, allowing a deeper understanding of the interplay between tumors and the immune system. Antibodies targeting the co-inhibitory receptors cytotoxic T lymphocyte-associated protein 4 (CTLA-4) and programmed cell death protein 1 (PD-1) have shown an impressive success in prolonging the survival of patients with various tumors including metastatic melanoma and lung cancer, at times bringing patients into long-term remission. However, release of these so called “checkpoint inhibitors” has led to the emergence of inflammatory and autoimmune-like reactions that have been predicted from gene knockout animal studies over a decade ago. While checkpoint inhibitors have been successful in a number of

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malignancies, there has been less success in other tumors, while others are undergoing clinical trials. In this regard, glioblastoma (previously glioblastoma multiforme, GBM) is the most common primary brain tumor of the adult, and carries a grave prognosis. Patients with glioblastoma develop a profound systemic immunosuppression, thought to be a hallmark of the failure of the immune system in rejecting the tumor. Therefore, given the limited therapeutic approaches available for management of glioblastoma, strategies aimed at counteracting the immune dysfunction and ultimately blocking tumor growth are being actively pursued. Nevertheless, given the limited self-renewal capacity of the central nervous system (CNS), autoimmunity in the CNS can have serious sequelae. Therefore, application of checkpoint immunotherapy for glioblastoma requires a deep understanding of how co-inhibitory receptors regulate both tumor immunity and self-tolerance.

1. T cell exhaustion

T cell exhaustion is defined as a dysfunctional state of T cells characterized by: a progressive and hierarchical loss of an acquired effector program, high expression of multiple co-inhibitory receptors, poor responses to cytokines promoting long-term survival, alterations in metabolism and use of key transcription factors (1). While most of the mechanistic insight on T cell exhaustion has been gathered with the help of animal models of viral infection, similar features have been described in the context of cancer. An immunological parallel between these two conditions has been strongly supported by a study that has found an important degree of transcriptional overlap between human melanoma-antigen specific tumor-infiltrating lymphocytes (TILs) and virus-specific T cells in chronic lymphocytic choriomeningitis virus (LCMV) infection (2).

T cell exhaustion was first described in chronic LCMV infection in mice (3), where virus-specific CD8 T cells were unable to eliminate the virus; similar findings have been since described with human infections such as human immunodeficiency virus (HIV), hepatitis C virus (HCV) and cytomegalovirus (CMV), as well as in cancer. The most important driver of exhaustion is thought to be chronic exposure to cognate antigen; in fact, multiple reports have found a correlation between the degree of dysfunction and the duration of antigen exposure (4–6). Lack of CD4 help has also been reported to impair sustained effective anti-viral CD8 responses during chronic infection (7), an observation paralleled in HIV where deeper loss of CD4 T cells correlates with a more profound CD8 exhaustion. Secretion of interleukin (IL)-21 by CD4 T cells appears to be crucial for CD8 effector responses (8, 9) through induction of sustained expression of the transcription BATF, which cooperates with IRF-4 to maintain Blimp-1 expression, and as such CD8 effector functions (10). Exposure to type I interferons (IFN) has been proposed to have time-dependent opposing effects on exhaustion induction: indeed, while promoting CD8 effector differentiation at early time points in the course of an infection, chronic IFN β stimulation is detrimental to antiviral CD8 T cell responses. While the precise mechanism has not yet been determined, there are indications for an indirect role through CD4 T cell help (11, 12). It is important to stress that exhausted cells are not entirely inert towards the infection: indeed, their elimination from the antigen-specific pool might result in exacerbation of a dormant chronic infection. Therefore, exhaustion is thought to represent a co-evolutionary compromise between host and

pathogens, especially viruses that can enter a latency phase, in order to establish an impasse and prevent immunopathological damage (1).

Metabolism of exhausted T cells

Circulating naïve T cells are quiescent and predominantly use oxidative phosphorylation for their metabolic needs. With antigenic priming, T cells undergo a series of significant metabolic changes resulting in a switch to a highly glycolytic metabolism. While glycolysis is less energy-efficient than oxidative phosphorylation in terms of molecules of ATP synthesized, it is crucial for production of IFN γ . Indeed, Chang et al. reported that the glycolytic enzyme GAPDH has a dual function resulting in inhibition of IFN γ mRNA maturation. As such, a higher rate of glycolysis results in a higher involvement of the enzyme in this pathway, and consequent release of IFN γ mRNA to the translation machinery (13). The glycolytic switch of activated T cells is instructed by activation of the mammalian target of rapamycin (mTOR) pathway, which is initiated by TcR and CD28 signaling (14). mTOR activation directly induces expression of the glucose transporter Glut1 (15), promotes protein synthesis and other anabolic pathways, and operates a transcriptional program directly linked to the acquisition of specific effector functions and lineage specification. Formation of a memory T cell pool require another metabolic change: in fact, memory T cells sustain themselves through fatty acid oxidation, requiring a series of adaptations including increase in the mitochondrial mass (16). Nutrient scarcity can be a driver of T cell exhaustion in the tumor microenvironment, especially when tumor cells are competing with T cells for glucose and glutamine. In animal models of an implanted antigenic sarcoma that is usually rejected by the immune system, forcing the tumor cells to acquire a highly glycolytic metabolism through long-term culture in high glucose media prior to injection was sufficient to disable T cells from the ability to reject the tumor (17). T cells that are deprived of glucose also present an abnormal Ca²⁺ re-uptake in the sarco-endoplasmic reticulum, interfering with T cell receptor (TcR) signal transduction (18). Finally, catabolic by-products released by high turn-over of tumor cells like kynurenine can dampen proliferation of CD8 T cells and induce regulatory T cells (Tregs) via a mechanism dependent on the activation of the transcription factor aryl hydrocarbon receptor (AhR) (19).

Expression of co-inhibitory receptors

Stable expression of multiple co-inhibitory receptors is also a hallmark of exhaustion. Co-inhibitory receptors are a large and expanding family of molecules that tune and shape activating signals to ensure self-tolerance, lineage specification, and resolution of the immune response. Given their crucial functions in determining the fate of a T cell at multiple phases, they are also referred to as checkpoint receptors. Common traits of this functional class of molecules are the heterodomain competition with a costimulatory receptor for a shared ligand, as well as having intracellular domains with immunoreceptor tyrosine-based motifs (ITIM) and immunoreceptor tyrosine based switch motifs (ITSM), containing tyrosine residues that, when phosphorylated, recruit SH2-containing phosphatases and other adaptor proteins. Among the co-inhibitory receptors that are best functionally characterized and most relevant for cancer immunology are CTLA-4, PD-1, T-cell immunoglobulin and mucin domain-containing (Tim)-3, lymphocyte activation gene (LAG)-3 and T cell

immunoreceptor with immunoglobulins and ITIM domains (TIGIT); therefore, the structural and signaling properties of these receptors will be detailed here.

CTLA-4 is sequestered in a large pool of intracellular vesicles, and rapidly up-regulated on naïve T cells upon TcR-induced Ca^{2+} influx (20); its expression is maintained at high levels on Tregs and follicular T helper cells (Tfh). CTLA-4 outcompetes CD28 for interaction with B7.1 and B7.2 (21) by means of a greater affinity due to bivalent binding, which can result in removal of these molecules from the membrane of antigen-presenting cells via transendocytosis (22). The cytoplasmic domain of CTLA-4 has a YVKM motif that can be phosphorylated to recruit the phosphatases protein phosphatase 2 (PP2A) and Src homology region 2 domain-containing phosphatase (SHP)-2 (23). Ligation of CTLA-4 induces dissociation of PP2A, allowing for sequestration of phosphoinositide 3 kinase (PI3k), which strongly dampens CD28 signals through the PI3k/Akt/mTOR pathway. CTLA-4 signaling can also impact on the T helper (Th)1/Th2 balance through tuning of Jnk activity (24).

PD-1 is expressed by T cells, particularly Tfh, as well as B cells at the pro-B stage and macrophages. Absent from naïve T cells, PD-1 is induced within the first 24h of activation. The intracellular tail of PD-1 contains both an ITIM and an ITSM motif, but deletion experiments have revealed that the ITSM is superior to the ITIM in dampening T cell proliferation and cytokine production (25). Upon TcR activation, both motifs are phosphorylated by lymphocyte-specific protein tyrosine kinase (Lck) and Src kinases, to induce recruitment of the phosphatases SHP-1/SHP-2. Binding of PD-1 by its ligands PD-L1 (constitutively expressed on T cells, B cells and a variety of myeloid cells) and PD-L2 (inducible on myeloid cells) licenses the two phosphatase to counteract early TcR and CD3 signaling, as well as the CD28-mediated activation of PI3k/Akt/mTOR (26).

TIGIT expression is also induced upon activation, but with important variations in different populations of T cells, with Tregs and Tfh being the most highly expressing subsets. TIGIT shares a partially overlapping pattern of ligands with the co-stimulatory receptor CD226: both molecules recognize the nectin-like molecule CD155 (which is used by the poliovirus as a receptor), although, likewise CD28 and CTLA-4, TIGIT has an affinity for CD155 that is 30 times higher than the one of CD226 (27, 28). TIGIT and CD226 are also expressed by natural killer (NK) cells, where they have both been implicated in the formation of immunological synapses with target cells via binding to CD155, although TIGIT also inhibits delivery of signals that interfere with degranulation (29). Signal transduction by the intracellular ITIM domain of TIGIT has been studied in NK cells, where recruitment of growth factor receptor-bound (Grb) 2 and SH2 domain containing inositol-5 phosphatase-(SHIP)-1 has been reported to dampen the PI3k/Akt/mTOR and the mitogen-activated protein kinase (MAPk) pathways (30). Moreover, TIGIT recruits β -arrestin, resulting in down regulation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B) signaling (31). Of note, we have reported that in CD4 T cells, TIGIT signaling does not merely suppress global T cell activation, but induces a specific shift of the effector profile. Knock-down of TIGIT on human CD4 T cells, in fact, was found to increase production of IFN γ and IL-17 in a CD226-dependent manner, and concomitantly reduce IL-10 (32). CD226 is consistently up-regulated during induction of Th1 and Th17 cells, but not Th2 (33), and a critical role for TIGIT in promoting Th2 responses was reported in experimental

allergic disease, where blockade of TIGIT inhibited Th2 polarization *in vitro*, and conferred some protection from allergic airway disease *in vivo* (34).

Tim-3 is predominantly induced on T cells that secrete IFN γ , both in CD4 and CD8 T cells, although multiple subsets of innate immune cells including DCs, monocytes and NK cells can also express Tim-3. Indeed, expression of Tim-3 is induced gradually during the course of Th1 differentiation, following direct binding of the transcription factor T-box expressed in T cells (Tbet) on the Tim-3 promoter (35). Cells with scavenger functions can use Tim-3 to detect dying cells by means of a cleft-like structure in the extracellular domain that can bind phosphatidyl serine moieties exposed at the surface of apoptotic cells (36). Tim-3 binds its ligand galectin (Gal)-9 through oligosaccharide residues present on its immunoglobulin domain (37). Despite not having a defined ITIM motif, the intracellular domain of Tim-3 has five tyrosine residues that can be phosphorylated upon ligation. Interestingly, binding of Gal-9 can shift the function of Tim-3 from an activating to an inhibitory signal. It has been shown that when Tim-3 has not been cross-linked, its cytoplasmic tail binds HLA-B associated transcript (Bat)3, which sequesters the phosphatase SHP-2 and recruits the kinase Lck, thus contributing to the TcR signaling cascade (38). Recently, it has been clarified that, besides binding Gal-9, Tim-3 needs to heterodimerize with carcinoembryonic antigen-related cell adhesion molecule (CEACAM)-1 in cis and/or in trans in order to display an inhibitory function (39). The same intracellular binding site occupied by Bat3 can be bound by Fyn, a kinase that has been implied in T cell anergy (40). Therefore, the ratio between Bat3 and Fyn occupancy seems to be important in determining the net effect of Tim-3 signaling: through this mechanism, Tim-3 can provide an early boost to activation, while contributing to shut down the response at later stages, depending on the availability of its ligands (41).

Finally, LAG-3 has been first discovered as a molecule induced on activated CD4 and CD8 T cells as well as a subpopulation of NK cells. LAG-3 is structurally related to CD4: as such, it binds to major histocompatibility complex (MHC)-II, but with higher affinity, as previously described for other couples of co-receptors with opposite functions. Translocated to the cell surface 24h after activation, the intracellular domain of LAG-3 can be cleaved by tumor necrosis factor (TNF) α converting enzymes (TACE) to release a soluble form (sLAG-3), which also might contribute to its regulatory function (42). The signaling downstream of LAG-3 is still unclear, but it has been established that a unique KIEELE motif present in the intracytoplasmic tail is essential for its inhibitory function, which contrast TcR activation, with a specific effect on the cell cycle resulting from the blocking into G2 phase (43, 44). Recently, LAG-3 was proposed as a marker of IL-10-producing forkhead box protein (FoxP) 3⁺ T regulatory (Tr)1 cells (45), although whether LAG-3 signaling is necessary for IL-10 production remains to be established. The observation that LAG-3 knockdown also impacts the function of CD8 T cells and NK cells, none of which expresses CD4, has prompted the identification of alternative ligands for LAG-3. A candidate for this role has been indicated in the Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin (DC-SIGN) family member liver and lymph node sinusoidal endothelial cell C-type lectin (LSECtin), highly expressed in the endothelium of liver sinusoids (46). Moreover, Kouo et al. identified galectin-3 as an additional and tumor microenvironment-specific ligand for LAG-3, showing that this novel

interaction suppresses both activated antigen-committed CD8 T cells as well as plasmacytoid dendritic cells (47).

Transcriptional control of exhaustion

While no single transcription factor has been identified as a master regulator of the exhausted phenotype, a hallmark of this dysfunctional state is a context-specific variation in the use of “general” transcription factors, and a combinatorial effect of their activity (1). For example, CD8 T effectors co-express Tbet and eomesodermin (Eomes), whereas these transcription factors appear to be expressed in a mutually exclusive fashion in exhausted T cells. After the resolution of an acute viral infection, in fact, Eomes function is important for the formation of a memory pool, by means of overseeing the return to a quiescent state and the homeostatic turnover of memory cells (48). However, in the case of a chronic infection, Eomes becomes responsible for the formation of an exhausted T cell pool. Specifically, two populations can be identified, a smaller pool of T-bet^{hi}PD-1^{int} cells, which retain some proliferative potential, and a larger pool of Eomes^{hi}PD-1^{hi} cells that show a more complete terminal differentiation and cannot generate long-lived memory T cells. Chronic antigen exposure stimulates the T-bet^{hi}PD-1^{int} population to proliferate and convert into Eomes^{hi}PD-1^{hi} T cells (49). A role for this transition was ascribed to the transcription factor forkhead box O (FoxO) 1, whose nuclear localization is promoted by PD-1 signaling, therefore establishing a positive feedback loop for PD-1 induction (50). Of note, a bias towards Eomes^{hi} CD8 T cells has been also found in HIV (51) and chronic HCV infection (49), with a positive correlation with more severe infection.

In order to shed further light on the relationship between activation and exhaustion, Anderson and colleagues have sought to discover gene modules that identify specifically exhausted cells and are not shared with activated cells. They profiled the transcriptome of TILs expressing combinations of Tim-3 and PD-1 from mice implanted with a melanoma cell line, and compared them to naïve and effector memory T cells from non-tumor bearing mice. This approach led to the identification of a cluster of genes enriched in Tim-3⁺PD-1⁺ TILs that contained a number of previously reported viral exhaustion signatures, but also some activation signatures. Genetic deficiency for the top-ranking gene in this cluster, the zinc chaperone MT1, was associated with a better tumor control in a T-cell intrinsic manner. Remarkably, MT1-deficient CD8 T cells revealed uncoupling of co-inhibitory receptor expression from T cell dysfunction, in that sustained expression of multiple co-inhibitory receptors did not mark dysfunctional T cells. Comparing the transcriptome of TILs from wild-type (WT) mice (activated and exhausted) and MT1^{-/-} mice (activated and not exhausted) highlighted a cluster of genes overexpressed in the WT group that had not previously been reported in transcriptional profiles of activation, and which was validated in a single-cell RNAseq dataset from human melanoma. The top-ranking gene in this signature was the transcription factor GATA-3, a crucial factor in Th2 differentiation, and also recently reported to promote induced Treg function (52).

In conclusion, while exhaustion develops as a consequence of T cell stimulation, the identification of the molecular control specific to exhaustion is of crucial importance to

develop therapies that allow for reversion or prevention of T cell dysfunction while preserving activation.

2. Glioblastoma

Generalities and etiology

Glioblastoma (world health organization, WHO, grade IV glioma) is the most common primary brain tumor of the adult. With an incidence of 5.6 per 100,000 adults in the USA (53), glioblastoma carries a poor prognosis, where patients receiving the standard of care have a median overall survival of only 15 to 17 months. These tumors tend to be more frequent in the cerebral hemispheres, often impairing cognitive functions, with a major impact on the quality of life. Since glioblastoma predominantly arises during the sixth to eighth decade of life, the tumor's prevalence is expected to increase with the prolongation of life expectancy. Whether glioblastoma develops *de novo* or evolves from a lower-grade astrocytoma, it is believed that the tumor results from a multistep process where cumulative genetic alterations ranging from point mutations to chromosomal aberrations disable multiple pathways involved in control of cell proliferation and identity (54). As a result, glioblastoma is characterized histologically by heightened cellularity and increased mitotic activity, neo-angiogenesis, central areas of necrosis and a marked invasiveness of the surrounding brain parenchyma. Recent work by Osswald and colleagues has revealed malignant astrocytomas forming ultra-long membrane projections that allow propagation across the brain, eventually leading to tumor recurrence at a new site (55). Nevertheless, glioblastoma is not known to induce extra-cerebral metastases, suggesting that its growth is dependent on micro-environmental factors specific to the CNS, although it is also possible that the short survival of patients with glioblastoma doesn't allow for metastasis detection.

Glioblastoma is slightly more common in men than women (sex ratio = 1.4) and in Caucasians more than in individuals of African descent (53). While gliomas can develop as part of the clinical picture of certain rare hereditary syndromes where onco-suppressors are mutated, such as neurofibromatosis and Li-Fraumeni syndrome, in the vast majority of cases there is no known causal association with a given genomic mutation. Genome-wide association studies for glioblastoma have identified a number of polymorphisms in genes involved in control of cell growth and proliferation, though as is the case for genetic variants associated with autoimmunity, each of these variants only contributes a minor increase in the total susceptibility (56). Among the environmental factors that have been considered as risk factor for glioblastoma, no association has been found for exposure to electromagnetic fields (including cell phones), head trauma and occupational exposures; these analyses have instead established that ionizing radiation is a risk factor for glioblastoma, although not at the level used in diagnostic procedures (57). A substantial amount of data, culminating with a recent report from the Glioma International Case-Control Study, has described an inverse correlation between allergies and glioma. A history of respiratory allergies conferred approximately 30% reduction in risk of developing a glioma, an association that is also confirmed when restricting to high-grade glioma (58). Respiratory allergy might represent a state of heightened immune surveillance at a site, the nasal mucosa, which is not only

important for the establishment of immune responses, but also permissive to the CNS penetration of certain substances or infectious agents through the trigeminal nerve.

It has been proposed that viral infections could contribute to the etiology of glioblastoma. Several groups, in fact, have investigated the presence of CMV antigens and nucleic acids in tumor resections. After a series of contrasting reports, a recent multi-centric publication has defined a standardized approach for detecting even low levels of CMV infection. Based on this standardized approach, it has been concluded that there is a consensus on the presence of this virus on most if not all glioblastoma tumors (59). The role of CMV in glioblastoma oncogenesis could be multifactorial: it has been reported that CMV infection leads to aberrant differentiation of neural precursors and upregulation of oncogenic proteins by glioma cell lines; moreover, CMV induces a well-described systemic immunosuppression of the host, reminiscent of the one observed in glioblastoma patients. Further research on the link between CMV and glioblastoma is needed in light of current and future therapeutic approaches that aim at inducing an anti-CMV immune response in the tumor.

Tumor biology

Many of the molecular alterations described in glioblastoma accumulate within certain fundamental cellular pathways involved in control of cell metabolism, genome integrity and consequent fate decisions on proliferation versus apoptosis. The Ras-MAPk pathway transduces pro-mitotic signals, driving entry into cell cycle. As previously mentioned, germline loss-of-function mutations in the Ras antagonist neurofibromin (NF)1 cause neurofibromatosis (60, 61), a syndrome that predisposes the individual to astrocytomas and secondary glioblastoma; but also 15–18% of primary glioblastoma harbor somatic mutations in the *nf1* gene (62). Among the receptor tyrosine kinases that deliver the signals of growth factors, the epidermal growth factor receptor (EGFR) is remarkable for a high level of genomic amplification, leading to more than 20 gene copies in more than 40% of tumors (63). It has been proposed that gene amplification of the *egfr* gene creates favorable conditions for the emergence of point mutations, particularly a truncation of the extra-cellular domain of the protein, EGFRvIII, that results in a constitutively activated receptor (64). Even if the EGFRvIII receptor is expressed only by a fraction of cells within the tumor, it is also thought to drive enhanced proliferation of negative cells by a paracrine mechanism, possibly based on secretion of IL-6 and leukemia inhibitory factor (LIF), which in turn induces up-regulation of wild-type EGFR (65).

The platelet-derived growth factor receptor α (PDGFRA) locus also undergoes gene amplification in 15% of glioblastoma; similar to EGFR, the gene amplification is associated with point mutations leading to a constitutively active form (66). PDGFRA signaling promotes aggressive tumor growth, probably through both autocrine and paracrine mechanisms. Tumor cells that engage in a high proliferative activity require to undergo a key metabolic change known as the Warburg effect (67). In this process, tumor cells derive energy from a high rate of glycolysis followed by lactic acid fermentation of pyruvate, instead of oxidative phosphorylation of pyruvate generated by a comparatively lower rate of glycolysis. The PI3k/mTOR pathway is the master regulator of the cellular metabolism, and as such responsible for the Warburg effect (68). Moreover, activation of this pathway drives

protein synthesis and other anabolic pathways, while promoting resistance to apoptosis. In glioblastoma, multiple hits favor hyperactivation of the PI3k/mTOR pathway, including gain of function mutations in catalytic and regulatory domains of the PI3k enzyme (15% of glioblastoma), and loss of function mutations of the phosphatase and tensin homolog (PTEN), the major negative regulator of the pathway (30%) (69). Confirming the importance of this onco-suppressor in grade II and grade III gliomas, PTEN expression is also down-regulated by epigenetic and miRNA-based mechanisms (70). Among the molecules that oversee the control of genome integrity, p53 plays a crucial role by allowing for DNA repair initiation, blocking the cell cycle at the G1/S transition and directing the cell towards apoptosis in case of irreparable DNA damage. As such, loss of function mutations in *p53* are a hallmark of many cancers, including gliomas. Indeed, 87% of glioblastomas present loss of function mutations or gene copy alterations of the *p53* locus (69). Similarly, the onco-suppressor retinoblastoma (Rb)1, also involved in retention at the G1 phase, is mutated in 78% of glioblastoma. To confirm the importance of the cell cycle checkpoint for tumorigenesis, the *CDKN2A* locus, which encodes both Ink4a and Arf proteins, which regulate p53 and Rb1, is often homozygously deleted in glioblastoma (54).

The molecules isocitrate dehydrogenase (IDH)1/2 link cell metabolism and epigenetic control of gene expression: in fact, these citric acid cycle enzymes are targeted by loss of function mutations that lead to abnormal amounts of 2-hydroxyglutarate, an inhibitor of α -ketoglutarate-dependent dioxygenases, including DNA and histone demethylases. Mutations of the codon 132 of IDH1 are found in grade II and grade III gliomas, probably leading to epigenetic deregulations that favor further progression toward grade IV. Consistently, the status of the IDH1 locus is considered a discriminator between primary glioblastoma (wild-type) and secondary glioblastoma evolved from lower-grade gliomas (mutated). IDH1 status also identifies tumors with a very distinct transcriptional profile, highlighting the importance of this mutation for the tumorigenesis process (54).

Despite the described common patterns of mutations across tumors, glioblastoma is also characterized by a striking degree of intra-tumoral heterogeneity. The term “multiforme” was originally adopted because of the high variability in size and shape of the tumor cells. Recent studies have highlighted that such histological pleomorphism corresponds to a high level of transcriptional heterogeneity. Specifically, Patel and colleagues have recently published the first molecular dissection of glioblastoma at the single cell level (71). This analysis revealed that, while previous genome-wide expression studies had classified glioblastoma into four categories, namely classical, mesenchymal, proneural and neural, the individual tumors studied presented subpopulations enriched for each of the four signatures. The level of class heterogeneity was established as a negative prognostic factor. Moreover, analysis of single cells allowed for the identification of subgroups of cells displaying the hallmarks of a very active cell cycle, but also niches of cells that scored low for the proliferation gene module and were enriched for a signature of quiescence. Of note, such dormant cells are likely to be refractory to chemotherapy, proving the clinical importance of the findings uncovered by single-cell transcriptomics. Finally, it has been proposed that glioblastoma contain a subpopulation of self-propagating cancer stem cells, possibly derived from tissue-resident progenitor cells, that are responsible for tumor growth (72). Indeed, it has been reported in mouse models that tumor re-growth after alkylating chemotherapy was

dependent on a population of cancer stem cells (73). In the previously described single-cell transcriptome of glioblastoma, a stemness signature was derived by comparison of more vs less differentiated glioblastoma primary cell lines, and used to rank single cells. This analysis did indeed reveal a stemness gradient, which was negatively correlated with a proliferation signature. This finding is consistent with other reports that cancer stem cells divide at a slower rate, and are therefore more resistant to standard therapies targeting highly proliferative cells.

Current therapeutic strategies

The first line of intervention for newly diagnosed patients with a glioblastoma is maximal safe resection of the tumor, which, on top of relieving compression-related symptoms, can improve overall survival, provided that a threshold of extent of resection is met (74). Surgery is usually complemented with radiotherapy, in the form of either whole brain radiation or stereotactic radiosurgery. Consistent with the highly infiltrative nature of glioblastoma, there is currently no clear evidence for preferring the latter over the former (75). Coupling radiotherapy with the alkylating agent temozolomide (TMZ) has been found to extend the two-year survival rate from 10% with radiotherapy alone to 27% (76); nevertheless, this combination approach was shown to be effective only for patients harboring tumors with a dysfunctional O⁶-methylguanine-DNA methyltransferase (MGMT). Because MGMT reverts the genotoxic lesions provoked by TMZ, hypermethylation of the *mgmt* gene, which abrogates its expression, has been established as a predictive marker of therapeutic benefit from TMZ (77). Bevacizumab[®], a humanized monoclonal antibody targeting vascular endothelial growth factor (VEGF), a crucial pro-neoangiogenesis molecule, is currently undergoing clinical trials as a supplement to chemo-radiotherapy. Approved for treatment of a series of metastatic cancers, preliminary results in glioblastoma have so far shown an increase in progression-free survival, but not in overall survival (78). Less conventional strategies that have also obtained FDA approval for the treatment of recurrent glioblastoma include carmustine biodegradable wafers, which are placed at the site of the resected tumor to release chemotherapy locally (79), and transcutaneous delivery of low-intensity alternating electric fields, aimed at disrupting the mitotic spindle of dividing cancer cells (80). In conclusion, current therapeutic avenues for glioblastoma have been focused on tackling tumor cell proliferation by multiple different angles, but the limited impact on overall survival prompts the development of new strategies.

3. Failure of immune rejection of glioblastoma

Systemic immunosuppression in glioblastoma

As previously mentioned, the T cell dysfunction that develops in chronic viral infections and in cancer appears similar, a parallel based on phenotypical and mechanistic findings, and one that has allowed for important advances in both fields. Nevertheless, an important distinction between the two conditions relates to the nature of the largely unknown antigens recognized during anti-tumor immune responses, which may be neo-antigens, but also may be self-antigens, eliciting T cells with a lower affinity as they have undergone thymic selection. Moreover, it is possible that the priming of anti-tumor responses occurs in a less inflammatory environment, although this is difficult to provide evidence for in human

settings, and extensive cell death that accompany the fast-rate growth of some tumors can be a source of non-infectious danger signals for priming (81).

Patients with glioblastoma present with a systemic immunosuppression. Initial studies reported a profound CD4 depletion in subjects with glioblastoma pre-resection, sometimes consistent with the threshold established for acquired immune deficiency syndrome (AIDS) definition. Functional findings of immune dysfunction included cutaneous anergy to common bacterial antigens, lymphopenia and inability to mount delayed-type hypersensitivity responses to neoantigens or recall antigens, impaired *in vitro* proliferation of PBMCs in response to T cell antigens, and reduced antibody production in response to T-dependent B cell mitogen (82). Immunosuppression was found to correlate with tumor size but not location, and to be characterized by multiple defects in early TcR/CD3 signaling. Moreover, typical interventions that rescue anergy, such as IL-2 supplementation, failed to correct the immune defect of patients with glioblastoma. More recently, it was reported that within the diminished CD4 compartment of glioblastoma patients, Tregs were present at an increased frequency, correlating with CD4 T cell dysfunction; importantly depletion of the Treg fraction *in vitro* rescued the defects of the CD4 compartment (83). Despite such a profound systemic immunosuppression, there is a conspicuous inflammatory infiltrate in glioblastoma, often requiring the pre-operative use of steroids to reduce the resulting edema. It has been proposed that the size of the T cell infiltrate correlates with overall survival, despite the fact that the studies reporting such finding are small and mostly retrospective (84). Taken together, the general features of the immune system in glioblastoma patients are suggestive of a massive attempt by the immune system to reject the tumor, resulting in failure and immune exhaustion (Fig. 1).

The role of the tumor microenvironment

While the blood brain barrier protects the CNS from permeation of macromolecules, making it an important limiting factor for consideration of therapeutic options in glioblastoma, it does allow for circulation of activated T cells and other immune cells. Moreover, in glioblastoma the blood brain barrier is often disrupted because of an ongoing neo-angiogenic process. These considerations are important to the understanding of the conditions for the establishment of an immune response against glioblastoma antigens. Of note, a recent report has established that in 20% of patients with glioblastoma circulating tumor cells can be found in the peripheral blood, a result not influenced by surgical intervention (85). It is of crucial importance to determine whether such dissemination of glioblastoma tumor cells can also happen via the newly-identified dural lymphatic vascular system (86), and whether priming of the anti-tumor immune response could therefore happen in conventional secondary lymphoid organs. A transcriptional profiling of the myeloid compartment of the immune infiltrate in glioblastoma has recently been completed, revealing that tumor infiltrating myeloid cells cluster with un-polarized M0 macrophages, probably indicating heterogeneous populations distributed along a continuum between pro-inflammatory M1 and anti-inflammatory M2 lineages. It was also reported that myeloid cells in glioblastoma are mostly composed of CNS-resident microglia and myeloid-derived suppressor cells (MDSCs), and only marginally of peripheral macrophages (87). MDSC are a heterogeneous myeloid population with features of both granulocytes and monocytes,

which display an important T cell suppressor activity in different pathological contexts, but are particularly expanded in cancer (88). Collectively, these findings depict a myeloid landscape unfavorable to the efficient priming of tumor-specific T cells or their reactivation if priming happened in the periphery.

Multiple cell types in glioblastoma can secrete the immune suppressor cytokine TGF β , which is found at elevated levels in the serum of patients with glioblastoma, and has been proposed as the major driver of Treg proliferation. Moreover, other cytokines produced in the tumor microenvironment include IL-10, prostaglandin E-2, and the enzyme IDO (89). Glioblastoma could also induce T cell dysfunction by triggering co-inhibitory receptors. Diffuse or fibrillar expression of PD-L1 has been found in 88% of tumors from newly diagnosed patients and 75% of patients with recurrences. Of note, PD-L1 expression has been reported at much lower levels for other tumor types, and is near-absent in the brain parenchyma surrounding the lesion. Nevertheless, no correlation was found between PD-L1 expression and survival rate, nor between PD-L1 expression and the size of the tumor infiltrate (89). A recent report links PD-L1 expression by tumor cells to metabolic competition with infiltrating T cells. In fact, in a mouse model of implanted sarcoma, blocking PD-L1 on tumor cells was sufficient to reduce mTOR activity and glycolysis rate of tumor cells regardless of PD-1 binding (17). While such function for PD-L1 will require confirmation in glioblastoma, biopsies have shown a heterogeneous pattern of metabolic activity, with glycolysis-associated enzymes and transporters enhanced in areas of hypoxia compared to the invasive edge, where oxygen tension might be normal, and respiration possible (90). Therefore, in the case of glioblastoma a more complex regional analysis of PD-L1 expression, metabolic activity, and T cell infiltration might be necessary to assess whether tumor cells establish a nutrient-deprived milieu via intrinsic PD-L1 signaling.

Finally, the interaction between the immune system and cancer stem cells is still largely unresolved, but *in vitro* studies have shown that these cells can repress cytotoxic responses, induce s, modulate macrophage/microglia functions and directly secrete IL-10 and tissue growth factor (TGF) β (72). Of note, TGF β autocrine signaling is a key factor for self-renewal of this cell population. These results therefore strongly suggest a major role for cancer stem cells in creating an immunosuppressive microenvironment at the tumor site.

The Role of Tregs

In addition to being increased in the periphery, Tregs infiltrate glioblastoma, a feature shared with other cancers such as melanoma, non-small cell lung cancer (NSCLC), gastric and ovarian cancer (91). Moreover, in glioblastoma high frequencies of intra-tumor Tregs have been established as a predictor of poor prognosis (92). It has been proposed that the tumor recruits Tregs from the periphery through secretion of the chemokines CCL22 and CCL2, which could be induced by hypoxic conditions (93). Increased migration of Tregs at the tumor site could also be a result of preferential activation of Tregs over T effector cells. In fact, it has been reported using both implanted and spontaneous tumor mouse models that memory Tregs are the first T cell subset to be exposed to tumor antigens. This advantage was related to the memory status of self-specific Tregs rather than their frequency, linking maintenance of self-tolerance to inability to reject tumors (94). Additionally, it has been

proposed that T effector cells could be redirected to the regulatory lineage by tumor-produced TGF β (95). Indeed, TGF β signaling in the presence of IL-2 is a well-established stimulus that can induce FoxP3 expression in mouse naïve T cells, by means of direct binding of TGF β -activated SMAD2 and SMAD3 to the *FOXP3* locus (96). Nevertheless, FoxP3⁺ Tregs induced with TGF β in humans fail to display suppressor activity *in vitro*, and the role of such stimulation for the generation of FoxP3⁺ cells with regulatory properties *in vivo* has been questioned (97). Another layer of complexity was added by a study reporting that glioblastoma-infiltrating Tregs are mostly of thymic origin based on co-staining of FoxP3 and the transcription factor Helios (98), but the reliability on expression of Helios for unequivocal identification of thymic Tregs (99) has also been recently questioned (100).

We recently published the first functional characterization of Tregs infiltrating glioblastoma, reporting the unexpected finding that tumor-derived Tregs are functionally impaired compared to circulating Tregs from the same individual (101). Tregs were identified based on standard markers (CD4⁺CD25^{hi}CD127^{lo}) from tumor infiltrates and blood of newly diagnosed patients, and their functionality was measured by assessing their capacity to suppress the proliferation of autologous circulating T effector population (CD4⁺CD25^{lo/int}CD127⁺) derived from the blood. We observed a significant reduction in the suppression activity of Tregs isolated from the tumor. This defect was associated with enhanced production of IFN γ by tumor-derived Tregs, as assessed after *in vitro* stimulation through TcR and CD28. Strikingly, this Th-1-like phenotype of Tregs was previously reported by others and us in multiple sclerosis (MS) and in patients with type 1 diabetes, and directly linked to Treg dysfunction (102, 103). The biological relevance of pro-inflammatory cytokine production by Tregs is unclear, and it has been suggested that it could be acquired alongside Th1-associated chemokine receptors that are needed for Tregs to relocate to the same sites where Th-1 responses take place (104). Nevertheless, a pathologic association for IFN γ production by Tregs has been suggested by the fact that this feature is acquired by CNS-infiltrating Tregs at the peak of experimental autoimmune encephalomyelitis (EAE) (105) and that in patients with MS the frequency of Th1-like Tregs is reduced by treatment (102). Tregs isolated from the tumor bed of patients with glioblastoma also expressed high levels of PD-1 and Tim-3 compared to the blood, as will be further detailed in the following sections, and this was most pronounced in glioblastoma as compared to gliomas of lower WHO grade. The surprising finding of reduced suppressor activity of tumor-infiltrating Tregs could indicate that they have become dependent on microenvironment-specific factors in order to perform optimal suppression. Indeed, as previously mentioned, it has been reported that the regulatory phenotype induced by TGF β stimulation is unstable. Moreover, while the choice of performing suppression assays with T effectors derived from the blood allowed us to directly compare tumor-derived and blood-derived Tregs, a question that still needs to be addressed is the possibility that the residual suppressor function of tumor-derived Tregs is sufficient for inhibition of tissue-resident T effectors. Concerning the Th1-like phenotype acquired by tumor-derived Tregs, it is possible that this reflects acquisition of a migratory profile allowing for infiltration of inflamed sites. It is also still unclear whether in glioblastoma effective immune responses fail to be generated because of inadequate priming conditions, or whether they are mounted, but subsequently repressed. The finding of IFN γ -producing Tregs is consistent with a scenario of proper establishment of a Th1/Tc1

effector response, leading to recruitment and/or local induction of Th1-like Tregs, which in turn contribute to dampening these responses, synergizing with micro-environmental factors and other suppressor cell populations.

4. Checkpoint receptor immunotherapy

Clinical success in other tumors

The first checkpoint receptor inhibitor to enter cancer immunotherapy has been a monoclonal antibody against CTLA-4, Ipilimumab[®], approved in 2011 for use in unresectable or metastatic melanoma. Monoclonal antibodies against PD-1 (Nivolumab[®] and Pembrolizumab[®]) followed shortly, with approval in 2014 for unresectable or metastatic melanoma. Nivolumab[®] has since been approved also for NSCLC, and has shown some therapeutic benefits against Hodgkin lymphoma and renal cell cancer (106–110). Despite their efficacy in the management of advanced malignancies, these drugs still have a therapeutic benefit in only a subset of patients, highlighting the need for biomarkers that can be used to predict treatment response. Studies in melanoma and lung cancer reported a degree of correlation between expression of PD-L1 and clinical response, but clinical activity has also been observed in PD-L1 negative tumors (111). Whole exome sequencing approaches have been used to explore whether the load of neoantigens can be such predictors. In melanoma, it was found that mutational load is indeed associated with clinical response, and specific resolution of a neoantigen landscape allowed for identification of strong responders, who harbored T cells that reacted *in vitro* to the neo-antigens that had been predicted with this approach (112). Likewise, in NSCLC, it was confirmed that higher non synonymous mutational burden is associated with improved and durable clinical response to PD-1 blockade and overall survival (107). Interestingly, an association was also found with a molecular signature regrouping smoking-induced mutations.

A recent study in melanoma has reported that increased frequencies of tumor-infiltrating PD1^{hi}CTLA-4^{hi} CD8 TILs pre-treatment were found to correlate with response to anti-PD-1 therapy and progression-free survival. These cells were described as being in a partially exhausted state, characterized by production of similar amounts of IFN γ and IL-2 as PD-1⁻ T cells, but less TNF α . Moreover, assessment of tumor infiltrating CD8 T cells in metastases developed during α PD-1 therapy revealed a partial correction of the dysfunctional exhausted state. Despite limited in size, this report paves the way for studies where expression on PD-1 on TILs is linked to function, and these parameters are reassessed over the course of treatment in relationship to clinical efficacy (113).

Role of checkpoint receptors in Treg function

The role of coinhibitory receptors for human Treg function is under active investigation. A study comparing the effect of CD3 stimulation coupled with multiple co-stimulatory and co-inhibitory receptors, analyzed individually, identified a similar hierarchy needed for costimulation signaling. Anti-CD28 emerged as the most important co-stimulatory signal for both Tregs and T effector, but inducing distinct effects in the two cell types. For example, at early time points some genes were equally induced in both, such as *tbx21*, but progression of the stimulation highlighted differences, including a T effector specific induction of cell-

cycle. The stimulation of Tregs with anti-CD3 plus anti-PD-1 or anti-CTLA-4, instead, revealed minimal effects for these molecule, confirming that in Tregs they likely function by shaping additional signals rather than the TcR alone (114). The *CTLA4* locus, a target of FoxP3, is constitutively expressed on FoxP3⁺ Tregs. Signaling through CTLA-4 is important for inducing partial Treg anergy in response to TcR and anti-CD28 stimulation. Moreover, the protein kinase C (PKC) η was shown to be recruited to the immunological synapse by the intracellular domain of CTLA-4 in Tregs, and this interaction proved important for contact-dependent suppression in tumor models, but not models of autoimmunity (115). Tregs derived from CTLA-4 deficient mice retain suppressive activity upon adoptive transfer in EAE and colitis models (116, 117). While conditional knockout mice lacking CTLA-4 expression on Tregs die of lymphoproliferative disease within one month of life (118, 119), a transgenic system allowing for Treg-specific CTLA-4 deletion during adulthood surprisingly revealed that Tregs displayed intact suppressive activity and were expanded. These findings suggest that CTLA-4 might be crucial for Treg selection and seeding during the neonatal phase, but then serve in adulthood to restrain and shape Tregs expansion in a similar way as it does for effector T cells (120). With regards to Ipilimumab[®] immunotherapy, it has been reported that CTLA-4 blockade induces antibody-dependent cellular cytotoxicity (ADCC)-dependent depletion of Tregs, rather than modulation of function, which could explain its high immune-related toxicity (121). Therefore, the observation that disrupting the CTLA-4:PKC η interaction disables suppression of tumor immunity but preserves control of autoimmune reactions (115) could prompt the development of non-depleting α CTLA-4 antibodies for cancer immunotherapy. Nevertheless, the finding that CTLA-4 blockade causes Tregs expansion highlights the importance of developing strategies to tackle only specific downstream effects of this pleotropic Treg molecule.

TIGIT is highly expressed on murine Tregs as well as human Tregs, even at the naïve state. The *TIGIT* locus is hypomethylated in Tregs and its transcription directly activated by FoxP3 binding (122). Sorted circulating TIGIT⁺ Tregs have been characterized molecularly and functionally by us and others, and found to have increased suppressor activity compared to the TIGIT⁻ Tregs. Moreover, we reported that TIGIT⁺ Tregs can inhibit Th1 and Th17 responses in both mouse and man, while sparing Th2 T cells (123). This pattern mirrors the T cell-intrinsic role of TIGIT, which is required for dampening the production of IFN γ and IL-17 in a CD226-dependent manner, but not Th2 cytokines. Moreover, TIGIT ligation induced IL-10, also reflecting a parallel situation for the function of this molecule between T effectors and Tregs. TIGIT⁺ Tregs were found to achieve the differential regulation of T cell responses through to the secretion of the soluble mediator fibrinogen-like protein (Fgl) 2, which regulates APC function through binding of FC γ RIIB. Moreover, Tregs can use TIGIT to shape antigen presenting cell (APC) profiles also through its capacity to bind CD155 on the surface of dendritic cells (DC)s, leading to repression of IL-12 production and induction of IL-10 (27).

While the function of LAG-3 for human Tregs is still being investigated, this receptor is expressed by both natural and induced Tregs in mice. It has been reported that blockade of LAG-3 can abrogate Treg suppressor function both *in glioblastoma* and *in vivo*, while enforcing LAG-3 expression on FoxP3⁻ CD4 T cells rendered them suppressive, possibly

through IL-10 production (124). Moreover, a specific role has been proposed for expression of Lag3 by Treg in restraining homeostatic expansion of T effectors in lymphopenic conditions (125).

PD-1 has been reported to be highly expressed in mouse Tregs, but in human PD-1⁺ Tregs only represent about 5% of the total circulating Treg population. In mouse, PD-1 signaling has been proven crucial for stable peripheral induction of s. Indeed, PD-1 stimulation contrasts activation of the PI3k/Akt/mTOR pathway through recruitment of the phosphatases SHP1/2 and PTEN, leading to nuclear translocation of un-phosphorylated FoxO1, where it activates a regulatory transcriptional program notable for stabilization of FoxP3 expression and of repression of IFN γ (126). Surprisingly, we found that in healthy humans, high expression of PD-1 marks Tregs that have lost their suppressor activity. PD-1 as a marker of dysfunctional Tregs was also reported in liver-infiltrating Tregs from patients with hepatitis C, which displayed impaired *in vivo* expansion and suppression activity through the interaction between PD-1 and PD-L1, resulting in blockage of signal transducer of activated T cells (STAT) 5 activity (127). Moreover, we detected elevated production of IFN γ upon *in vitro* stimulation of PD-1⁺ Tregs (101). IFN γ is thought to disable Treg function in an autocrine/paracrine loop, and we have previously reported that antibody blockade of IFN γ during *in vitro* suppression assays restores Treg function (102, 128, 129). Nevertheless, the same approach failed to rescue the functionality of PD-1⁺ Tregs, suggesting a more complex mechanism for their defect in function. Transcriptomic analysis of sorted circulating PD-1⁺ and PD-1⁻ Tregs revealed that these PD-1 positive cells express other co-inhibitory receptors, including Tim-3 and LAG-3, and display signatures of exposure to IFN γ and IL-12, consistent with our functional findings. Moreover, this analysis also highlighted an enrichment in pathways regulating progression through cell cycle, a finding that we interpreted as a transcriptional footprint of high proliferative activity *in vivo*. To confirm this hypothesis, we found that PD-1⁺ Tregs have eroded telomeres, and have a particularly low proliferative response to *in vitro* stimulation. Collectively, their dysfunctional state coupled with co-expression of multiple co-inhibitory receptors and hallmarks of previous chronic stimulation lead us to conclude that in man, PD-1 expression in the circulation marks a population of partially exhausted Tregs, possibly derived from IFN γ -producing Th1-like Tregs induced in inflammatory contexts. Finally, we observed that sorted circulating PD-1⁺ Tregs have higher phosphorylation of FoxO1 compared to the PD-1⁻ fraction, consistent with their capacity to secrete IFN γ . In this regard, a recent study indicates that activated Tregs actually need to repress FoxO1 in order to acquire appropriate migratory properties (130), suggesting that PD-1⁺ Tregs might be recirculating from tissue sites.

Since we also observed that that PD-1⁺ Tregs display a lower demethylation of the *FOXP3* locus than PD-1⁻ Tregs, reminiscent of the difference between induced and naturally occurring Tregs, we speculate that PD-1⁺ Tregs might be peripherally induced Tregs, where PD-1 signaling might have served for induction, as previously reported in mice (126). As previously mentioned, we report an increased frequency of PD-1⁺ Tregs in the glioblastoma tumor bed. Direct comparison of the transcriptome of circulating PD-1⁺ Tregs from healthy donors and glioblastoma patients identified very few differentially expressed genes, suggesting that PD-1 marks circulating Tregs with similar properties in the two conditions. When we further compared blood-derived versus tumor-infiltrating PD-1⁺ Tregs of patients

with glioblastoma, we observed that multiple signatures of activation, IFN γ exposure and exhaustion were enriched in the tumor. Functional clustering of the genes that were mostly co-expressed with PD-1 in PD-1⁺ Tregs from the tumor confirmed hallmarks of antigen exposure and pro-inflammatory cytokine signaling, consistent with the functional finding described above. Therefore, we propose a continuum of functional states of Tregs, where induction of Th-1-like markers and elevated antigen stimulation leads to progressive exhaustion of functional properties. PD-1⁺ Tregs in the circulation are in an intermediate state, whereas PD-1⁺ Tregs in the tumor bed represent a more extreme condition.

Tim-3 has been reported to be highly expressed by Tregs at tissue sites in various pathologic contexts, in mice as well as in human cancer. Indeed, we also report an enrichment in Tim-3 expression by tumor-infiltrating Tregs in gliomas of the highest WHO grades (101). Tim-3⁺ Tregs are absent from the circulation of healthy individuals, as well as infrequent in secondary lymphoid organs of mice, suggesting that this molecule might be important specifically for suppressor activity in the tissue as compared to homeostatic functions. In patients with glioblastoma, we found a small but detectable percentage of Tim-3⁺ Tregs in the circulation, also expressing PD-1, further strengthening the hypothesis that PD-1⁺, and even more so PD-1⁺Tim-3⁺ Tregs found in blood are a migratory population that recirculates from tissue sites. Finally, we observed that Tregs that acquire Tim-3 expression *in vitro* display enhanced suppressor activity towards Th1 and Th17 T cell responses, similar to what we described for TIGIT, together with increased secretion of IL-10 and “Tc1-like” molecules such as perforin and granzymes.

In conclusion, co-inhibitory receptors play a multi-faceted role on Tregs function, and further research is required to dissect the importance of this class of molecules for natural versus induced Tregs in the periphery as well as in the tissue.

Role of checkpoint receptors for maintenance of self-tolerance

Though elevated and stable expression of multiple co-inhibitory receptors is a hallmark of T cell exhaustion, the importance of this family of immunoregulatory molecules at earlier phases of T cell responses is evident due to the autoimmune responses developing with loss of function. In humans, this evidence comes directly from the secondary effects of checkpoint immunotherapy, that was predicted by earlier animal models of autoimmune diseases. Specifically, germline gene deficiency for PD-1 and CTLA-4 can cause fatal autoimmunity even in non-susceptible genetic backgrounds, resulting in lupus-like arthritis and glomerulonephritis in PD-1^{-/-} C57Bl/6 mice (131) and antibody deposition-induced cardiomyopathy when PD-1 was absent from the BALB/c background (132). More significant inflammatory responses are seen with CTLA-4^{-/-} mice where a massive lymphoproliferative disease leading to multi-organ tissue destruction, results in an early death (118, 119). The surprising observation that genetic loss of CTLA-4 during adulthood conferred a complete resistance to EAE (120) demonstrate the opposing roles of this molecule at different stages of development. The role of PD-1 was also explored in EAE where the interaction between PD-1 and PD-L1 (133) but not PD-L2 (134) axis in regulating CNS autoimmunity is critical. LAG-3 deficiency, instead, can also lead to autoimmunity, but only in predisposed genetic backgrounds, such as non-obese diabetic (NOD) mice, where

spontaneous emergence of diabetes was accelerated (135). Antibodies against Tim-3 administered during EAE worsened disease (136), whereas blocking its ligand, Gal-9, resulted in a milder clinical course (37): these two complementary observations therefore reveal that Tim-3 function contributes to the outcome of autoimmunity. Consistent with the previously described function of Tim-3 in regulating IFN γ -producing T cells, this effect was only seen in Th-1-induced EAE and not in models relying on Th17-polarized myelin-reactive T cells (137). Furthermore, reduction of Tim-3 expression has been reported in the peripheral blood of MS (138, 139) and rheumatoid arthritis patients (140), and induction of Tim-3 has been observed specifically in responders but not non-responders to IFN β therapy (141). TIGIT^{-/-} mice do not develop spontaneous EAE, but a worsened clinical course when disease was induced upon immunization with myelin antigens (142). Similarly, antibodies blocking TIGIT worsen ongoing EAE, and TIGIT^{-/-} 2D2 mice, which carry a myelin oligodendrocyte glycoprotein-specific TcR, develop a spontaneous atypical disease, reminiscent of disease induced by adoptive transfer of pre-activated Th17-polarized cells (143). Finally, to support the relevance of the TIGIT pathway for human autoimmunity, polymorphisms in the *CD226* gene (Gly307Ser) and *CD155*, were found associated with multiple autoimmune diseases including MS in genome-wide association studies (144, 145).

Autoimmune-like adverse reactions

It has long been known that blocking CTLA-4 co-stimulatory pathways with gene knockdowns or monoclonal antibodies can result in autoimmune, inflammatory diseases. Thus, it is perhaps not surprising that check-point inhibition used to treat cancer may result in autoimmune disease. A recent work by Ken Smith and colleagues provided an elegant transcriptional demonstration of this concept. By performing RNAseq transcriptomic profiling of CD8 T cells from patients with autoimmunity, they discovered that LCMV-derived CD8 T cell exhaustion signature identified a group of patients with a more benign form of autoimmune disease, indicating that the same mechanisms associated with T cell exhaustion are important in controlling autoimmunity (146). Consistent with this hypothesis, a number of immune-related adverse reactions have been described in checkpoint immunotherapy, including inflammatory and autoimmune events. We have recently characterized a case of CNS demyelination and enhanced myelin specific T cell responses after Ipilimumab[®] therapy. The patient had undergone Ipilimumab[®] treatment for metastatic melanoma, displaying a partial reduction of existing masses, but also a new lesion in the CNS requiring stereotactic radiosurgery. The patient subsequently developed neurological symptoms and MRI abnormalities, and a biopsy revealed inflammatory infiltrates and demyelination without signs of tumor cells. Myelin-specific T cell libraries (147) were generated and characterized for proliferation potential and production of inflammatory cytokines. The results from a previous comparison of multiple sclerosis patients and healthy controls were used as a reference (148). Strikingly, myelin-specific T cell libraries from the Ipilimumab[®]-treated patient presented the same pro-inflammatory bias of those from the MS patients, characterized by an increase in IFN γ , IL-17 and GM-CSF, and a decrease in IL-10 compared to healthy controls (149). A similar case had been previously reported, where a MS patient with stable disease underwent a relapse after starting Ipilimumab[®] treatment for melanoma (150). These and other cases have prompted a study of the safety of Ipilimumab[®] for advanced melanoma in patients with pre-existing autoimmunity, where 30% of patients

experienced a worsening of their conditions requiring systemic treatment with steroids. The study was not powered to assess a correlation between anti-tumor therapeutic efficacy and autoimmune exacerbations, which remains an open question (151). Ipilimumab® has also been reported to induce colitis, dermatitis, hepatitis and inflammatory endocrinopathies, sometimes with fatal outcomes (106).

Blocking of PD-1 is accompanied by similar immune-related toxicities, although less severe. Emergence of new-onset autoimmunity in the form of insulin-dependent diabetes was reported in five cancer patients receiving Nivolumab®, with pre-diabetes time spans as short as 1 week (and up to 5 months). Immunological findings associated with these adverse events included detection of anti-GAD65 antibody and antigen-specific CD8 T cells in patients with a given haplotype. Two patients also developed concomitant autoimmune thyroiditis (152). Therefore, interfering with CTLA-4 and PD-1 to reinvigorate anti-tumor T cell responses may induce serious autoimmune-like reactions.

Single or combination immunotherapy?

Functional analysis of co-inhibitory receptors suggests a degree of redundancy that may be tissue specific leading to a hierarchical model to explain the multiplicity of checkpoints for control of T cell responses (41). As depicted in Fig. 2, PD-1 and CTLA-4 may represent the first lines of self-tolerance with the fatal immune hyperactivation in knock-out animals, as well as the immune-related adverse reactions of therapeutic strategies targeting these molecules. TIGIT, Tim-3 and LAG3, appear to regulate more specific types of immune responses. For example, the ligands of Tim-3, CEACAM-1 and Galectin-9, are highly expressed in the gut (153), while the newly identified alternative ligand of LAG-3, LSECtin, is abundant in the liver, suggesting a tissue-specific role for these receptors. Likewise, CD155 expression has been characterized in the Peyer's patches (154) as well as in neurons (155), consistent with its role as receptor for the poliovirus. Moreover, the inhibitory function of TIGIT is specifically directed to Th1 and Th17 responses, sparing Th2 immunity. Moreover, mice deficient for these receptors display a milder phenotype. Thus targeting TIGIT, LAG3 and Tim-3 may allow a more organ specific strategy for cancer immunotherapy.

Based on these observations, it seems reasonable to vigorously pursue other co-inhibitory receptors as a therapeutic strategy. LAG-3 blockade was initially shown to enhance tumor vaccination in animal models (156), and to synergize with PD-1 in models of chronic infection (157). Phase-I studies of soluble LAG3-Ig in advanced renal cell carcinoma (158) and advanced pancreatic adenocarcinoma (159) showed that the drug is well-tolerated with little side effects, inducing a clinical response at the highest doses tested. Moreover, blocking antibodies for LAG3 have recently entered clinical trials. It has been proposed that TIGIT blockade could synergize with anti-PD-1 therapy. Indeed, tumor-infiltrating CD8 T cells from melanoma patients co-express high levels of TIGIT and PD-1, and co-blockade was superior to single agents in the induction of cytokine production and proliferation *in vitro* (160). In murine models, TIGIT also exhibited synergy with Tim-3, with control of tumor growth (161). Tim-3 and PD-1 co-expression has been reported in different animal models of cancer, as well as in human disease including our findings in glioblastoma.

Moreover, PD-1 and Tim-3 single or double expression defines a hierarchy of dysfunction of TILs in melanoma models, where the most profound impairment in cytokine production and proliferation was reported for PD-1⁺Tim-3⁺ T cells (52). Blocking both Tim-3 and PD-1 has also revealed a more effective strategy than blocking PD-1 alone in preclinical models.

These considerations highlight the opportunity of using combination therapy including anti-PD-1 and anti-Tim-3/TIGIT to elicit a better clinical effect while potentially reducing toxicity. Nevertheless, synergy in clinical benefit may be accompanied by increased side effects. Combination therapy has indeed been tried for Ipilimumab[®] and Nivolumab[®] in advanced melanoma (162). The two drugs were tested either concomitantly or sequentially, starting with Ipilimumab[®]. When used together, 50% of the patients developed severe immune-related adverse events, including hepatic toxicity, renal toxicity, gastrointestinal toxicity, pneumonitis and uveitis. Stopping treatment because of adverse events was necessary in 21% of the patients, while the patients treated sequentially with Ipilimumab[®] followed by Nivolumab[®] had adverse events comparable with the administration of each drug alone. However, the objective clinical response was estimated to be 40% in the concomitantly treated group versus 20% in the sequential group, indicating that for combination checkpoint immunotherapy the uncoupling of the clinical benefit from the side effects has not yet been achieved.

Finally, the opportunity of targeting multiple checkpoint receptors is suggested by a recent study showing that in both mouse models and patients with adenocarcinoma, resistance to anti-PD-1 therapy was associated with increased expression of Tim-3. Of note, in the animal model, blocking Tim-3 after failure of anti-PD-1 therapy was successful in enhancing rejection of the tumor (163). Thus, this study indicates that the partial functional overlap between co-inhibitory receptors can be exploited by tumor cells as an escape strategy, similar to resistance to chemotherapy.

5. Checkpoint immunotherapy for Glioblastoma

When considering the application of checkpoint immunotherapy to glioblastoma, issues related to the CNS localization of the cancer need to be taken into account. As previously mentioned, the blood-brain barrier is often disrupted in glioblastoma; nevertheless, it cannot be assumed that antibodies would effectively penetrate the brain parenchyma. Therefore, it is unclear if their mechanism of action would derive from an activity *in situ* or rather from functional modulation of peripheral T cell populations that recirculate from the tumor. Moreover, given the limited regenerative capacity of the neuronal tissue, adverse events from CNS inflammation of brain parenchyma would be particularly deleterious. The experience with Ipilimumab[®] and Nivolumab[®] in other cancers has not pointed to a clear pattern for which organs may be targeted by autoimmune-like reactions evoked by immunotherapy: indeed, both systemic and organ-specific events were reported with no clear link to the original site of the tumor. Nevertheless, our description of CNS demyelination after Ipilimumab[®] treatment and stereotactic radiosurgery in a patient with a brain metastasis might suggest careful monitoring for secondary autoimmune diseases in the CNS. Moreover, we and others have repeatedly reported that myelin-reactive T cells exist at similar frequencies in MS patients and healthy controls, and we have recently shown that

healthy individuals respond to self-antigen stimulation by secreting IL-10 rather than pro-inflammatory cytokines (148). Therefore, if this tolerant state is at least partly dependent on action of co-inhibitory receptors, checkpoint immunotherapy may break tolerance and allow myelin-reactive T cells to attack the CNS. It is also important to consider how checkpoint immunotherapy could complement existing therapies. Radiation causes extensive necrosis, which could be a source of neo-antigens. In some patients receiving stereotactic radiosurgery, an abscopal effect developed, defined as the shrinking of a tumor lesion separated from the one that has been irradiated, and which is thought to be immune mediated. Retrospective analyses in melanoma showed that radiotherapy and immunotherapy synergized to induce the abscopal effect, and the extent of this effect correlated with improved survival (164).

A question that remains open with regard to coupling immunotherapy with radiotherapy is whether the two should be administered concomitantly or consecutively. Chemotherapy with TMZ is associated with leukopenia and lymphopenia, raising the issue that concomitant use of these agents may result in less effective checkpoint inhibition. On the other hand, it has been suggested that lymphopenia may reduce Treg frequency eliminating homeostatic brakes to T effector cell proliferation (165, 166). Specifically, it is known that immune deficiencies can be accompanied by autoimmune and inflammatory reactions. Thus, although more studies are needed, it seems reasonable to establish regimens where immunotherapy complements existing therapies.

What checkpoint receptor(s) would be the ideal target in glioblastoma? In animal models of glioma, blockade of CTLA-4 and PD-1 has been tried with some success. Treating animals with established murine glioma with α CTLA-4 resulted in long-term survival in 80% of mice. Of note, EAE was not observed. The treatment corrected the tumor-associated Treg overrepresentation, but Treg function was preserved, possibly explaining the maintenance of tolerance to self despite enhanced anti-tumor immunity (167). Combination therapy with anti-PD-1, anti-Tim-3 and stereotactic radiosurgery has also been recently tested in mouse models of implanted gliomas. While each individual treatment as well as binary combinations prolonged survival to some degree, triple treatment resulted in long-term survival in 100% of the animals (168). An argument for combining PD-1 and Tim-3 blockade for glioblastoma immunotherapy comes from the observation that Tim-3 activity promotes MDSCs expansion (169), which, as mentioned before, are the most abundant myeloid population in the tumor infiltrate. Moreover, it has been reported that hypoxia-induced PD-L1 expression by MDSCs contributes to IL-10 production (170).

Nivolumab[®] and Ipilimumab[®] have recently entered clinical trials for glioblastoma. In a small phase I trial for Nivolumab[®] alone or in combination with Ipilimumab[®], we observed an increase in the IFN γ production by circulating Tregs of patients receiving the anti-PD-1 drug alone (100). This result is consistent with the role of PD-1 in blocking *IFNG* transcription through nuclear localization of FoxO1. Although the functionality of Tregs during Nivolumab[®] therapy was not assessed, and the study was not powered to establish efficacy, this result represents a proof of concept that PD-1 signaling represses Th-1 reprogramming of Tregs in glioblastoma, and thus targeting PD-1 could destabilize the regulatory program. Nevertheless, it should be kept in mind that IFN γ signaling induces PD-

L1 expression on tumor cells (171, 172), and that PD-L1 has been seen to sustain the metabolism of tumor cells even in the absence of PD-1 ligation (17). Therefore, the choice of the ideal target for immunotherapy will require a dissection of the effector mechanisms that are induced as part of the reinvigoration process, and of the interaction between pro-inflammatory cytokines and tumor cells in the tumor microenvironment.

Conclusion

The clinical success of checkpoint immunotherapy targeting PD-1 and CTLA-4 in advanced cancers represents a significant advance in cancer therapy, and has prompted an intense effort to extend this approach to other malignancies, including brain tumors. Mounting evidence suggests a crucial role for immune dysfunction glioblastoma, the most common primary brain tumor of adults. Indeed, patients with glioblastoma present with a profound systemic immune deficiency, associated with reduced CD4 counts and increased frequency of s. The immunoregulatory cytokine TGF β , considered a driver of peripheral Treg induction, is elevated in the serum of patients with glioblastoma, and it is thought to be secreted by the tumors, creating an immunosuppressive microenvironment. Moreover, the vast majority of these tumors express high levels of PD-L1, the ligand for the co-inhibitory receptor PD-1, as well as chemokines specifically sensed by s, resulting in accumulation of Tregs in the tumor infiltrate. Collectively, these findings suggest conditions favorable to checkpoint inhibition immunotherapy in disease management, though a number of key questions are unknown. First, while the importance of PD-1 and CTLA-4 has been demonstrated for induction of Tregs in the thymus and periphery, CTLA-4 seems to have a homeostatic function in restricting Treg expansion, whereas we report that PD-1 expression identifies circulating and tumor infiltrating Tregs with partially exhausted suppressor functions. Therefore, future research is needed to address the role of these co-inhibitory receptors with regards to Treg function in glioblastoma. Moreover, severe inflammatory and autoimmune-like reactions accompany anti-PD-1 and anti-CTLA-4 therapy, highlighting the importance of these pathways not only in regulating T cell exhaustion in cancer, but also maintenance of tolerance to self. We reported that anti-CTLA-4 therapy in a patient with brain metastasis of melanoma resulted in a demyelinating attack, during which the cytokine profile of myelin-reactive T cells was similar to what is reported in patients with MS. Thus, further research is required to achieve the induction of anti-tumor immune response without emergence of CNS-specific autoimmunity with checkpoint immunotherapy for brain cancer. Thus a class of co-inhibitory receptors representing the second line of checkpoints for immune activation, including Lag-3, Tim-3 and TIGIT, represent promising candidates. Indeed, these pathways are involved in regulation of more targeted, tissue-specific immune responses, and could display clinical benefit with less adverse reactions. Nevertheless, their role in dampening autoimmune responses was often demonstrated in animal models of CNS autoimmunity. In conclusion, future research is necessary to identify strategies to fine-tune the balance between T cell exhaustion and tolerance to self to achieve safe rejection of brain tumors.

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Figure 1. Mechanisms of immune dysfunction in glioblastoma. A model for induction of T cell exhaustion in the glioblastoma microenvironment illustrating the role of preferential attraction of s, conversion of T effectors into Tregs through TGF β secretion, engagement of PD-1 by PD-L1, and metabolic competition.

Figure 2. Role of different co-inhibitory receptors in control of autoimmunity and anti-tumor immunity. Blockade of different receptors elicits hierarchical levels of tumor rejection and autoimmune complications, as reflected by the universal versus specific inhibitory activity