T-cell immune responses in the brain and their relevance for cerebral malignancies

Paul R. Walker, Thomas Calzascia, Nicolas de Tribolet, Pierre-Yves Dietrich

Abstract

In order that cellular immune responses afford protection without risk to sensitive normal tissue, they must be adapted to individual tissues of the body. Nowhere is this more critical than for the brain, where various passive and active mechanisms maintain a state of immune privilege that can limit high magnitude immune responses. Nevertheless, it is now clear that immune responses are induced to antigens in the brain, including those expressed by cerebral malignancies. We discuss hypotheses of how this can occur, although details such as which antigen presenting cells are involved remain to be clarified. Antitumor responses induced spontaneously are insufficient to eradicate malignant astrocytomas; many studies suggest that this can be explained by a combination of low level immune response induction and tumor mediated immunosuppression. A clinical objective currently pursued is to use immunotherapy to ameliorate antitumour immunity. This will necessitate a high level immune response to ensure sufficient effector cells reach the tumor bed, focused cytotoxicity to eradicate malignant cells with little collateral damage to critical normal cells, and minimal inflammation. To achieve these aims, priority should be given to identifying more target antigens in astrocytoma and defining those cells present in the brain parenchyma that are essential to maintain antitumour effector function without exacerbating inflammation. If we are armed with better understanding of immune interactions with brain tumor cells, we can realistically envisage that immunotherapy will one day offer hope to patients with currently untreatable neoplastic diseases of the CNS.

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Immune recognition of antigens

The role of the immune system is to maintain the functional integrity of the host in the face of biological threats arriving from the exterior (pathogenic microbes), or from within (neoplastic changes). It is clear that the necessity for such a defense system is organism-wide, at least for non-expendable, non-self renewing tissues and organs such as the brain. The only caveat that may be raised is if there is an efficient non-immunological defense mechanism. For the CNS, the physical protection afforded by the skull and a certain physical isolation provided by the endothelial/cellular barriers of the brain may raise the threshold for the necessity of immune intervention. However, the substantial list of cerebral malignancies and infections with pathogenic neurotropic viruses illustrates that the CNS is far from totally protected.

The original conceptual framework for understanding the operation of the immune system was that it functioned by the discrimination of self from non-self. We are apparently ‘hard-wired’ with an innate immune system that detects certain basic patterns that are highly conserved amongst pathogenic organisms, and affords some immediate protection against infection by many microbes. The innate immune system includes complement components found in plasma, but in the interstitial tissue fluid (ISF) and cerebrospinal fluid (CSF) of the CNS, concentrations are very much lower. Cell-mediated innate immune responses include phagocytes bearing toll like receptors (TLR) that recognize conserved microbial-associated products such as lipoteichoic acid (on Gram-positive bacteria), mannans (on fungi) and lipopolysaccharide (on Gram-negative bacteria) [152]. In the CNS, the resident cells capable of mediating cellular natural immunity are the microglial cells, the macrophages of the brain. However, there are severe limitations to immune recognition by germline encoded receptors. Microbial pathogens evolve and mutate at a very high rate, generating an extraordinary number of variants that would soon exhaust the capacity of the host genome to encode a sufficient number of specific receptors to detect all microbial pathogens. Thus, although details of new receptors are regularly being reported, their number is probably in the region of a few hundred. Furthermore, innate immune responses are essentially the same at each re-exposure to infection; there is no specific immunological memory.

A complementary solution to immune recognition evolved in the jawed vertebrates: lymphocyte-based adaptive immune responses. A large number of T and B lymphocytes are generated in each individual, each bearing clonally distributed antigen receptors that are structurally related and are assembled following similar mechanisms of gene rearrangement. For both T and B cells, tolerance to self antigens is maintained by the elimination or regulation of autoreactive cells at different stages of their existence [175]. B cells can directly recognize free, native antigen, whereas T-cell antigen recognition is more complex, requiring an antigen presenting cell (APC). Within any given individual of a species, APCs display a selection of major histocompatibility complex (MHC) molecules that signal to the developing T cells a complex molecular signature corresponding to ‘self’, which normally stimulates no immune response, but which serves as a reference point for the same lymphocytes when later there is exposure to ‘non-self’ in the form of inevitable antigenic challenge by microbes. MHC molecules fulfil this role by sampling degraded proteins present within a cell and presenting peptides derived therefrom at the cell surface to T cells [88]. In the case of MHC class I molecules, which are expressed by most somatic cells (although notably, constitutive MHC expression by many cells of the brain is...
very low), these peptides are generally 8–10 amino acids in length and are contained in a groove in the MHC molecule that is closed at each extremity. The resulting MHC/peptide complex serves as the ligand for the T-cell receptor (TCR) of CD8 T cells. For MHC class II molecules, expression is normally limited to hematopoietic cells and thymic epithelial cells, although class II may be induced on other cell types by interferon-γ. The groove of MHC class II molecules permits binding of somewhat longer peptides than for MHC class I (most are 13–17 amino acids), since the extremities of the peptide are not anchored. The MHC class II/peptide complex is the ligand for the TCR of CD4+ T cells.

Because only few cells with a given fine specificity are present in an individual before antigen exposure, to achieve sufficient specific cells for meaningful biological function, lymphocytes must be appropriately stimulated to undergo clonal expansion. This takes a few days (during which time the innate immune response may be containing the pathogen), but the result is an army of effector cells expressing antigen receptors of identical specificity that may be able to eliminate the pathogen. A proportion of this expanded clone of lymphocytes is also committed to becoming memory cells, available for rapid response to future exposure to the same antigen, even many years later. In contrast, most of the effector cells are eliminated by apoptosis after elimination of the primary source of antigen [237,227].

A unique, genomically economical solution permits the adaptive immune system to generate a sufficiently large number of TCRs to bind virtually any conceivable antigen, without dedicating an unreasonable proportion of its genome to the task. This is achieved by somatic rearrangement of a limited number of gene segments, a process also employed in the assembly of the B-cell receptor for antigen, the immunoglobulin molecule. All TCRs are dimers, comprised mainly of an α and a β chain, but a minority population of T cells exists that expresses a γ and a δ chain. Only αβ TCR T cells will be discussed in detail here, since these cells recognize short peptides bound to MHC molecules as described above, whereas γδ T cells may have other ligands than classical MHC/peptide complexes. The mature αβ TCR is non-covalently bound to a cluster of invariant proteins, termed CD3, that are responsible for signal transduction. In addition, the TCR is associated with either a CD4 or a CD8 co-receptor, dictating MHC class II or MHC class I restriction, respectively.

Peptides that bind to MHC class I and class II molecules are generated by different pathways. For MHC class I binding peptides, they are most commonly considered to be of endogenous origin, i.e. self proteins and those encoded by viral genes or by genes mutated or over-expressed following neoplastic transformation. Thus, MHC class I expressing infected cells or tumor cells may potentially be detected and killed by CD8+ cytotoxic T lymphocytes. In contrast, the proteins that furnish the peptides that are assembled with MHC class II molecules typically arise from the extracellular milieu, for example bacterial antigens from strains that do not parasitize the intracellular compartment [131]. Despite this apparent dichotomy in the handling of endogenous and exogenous antigens, it has become apparent in recent years that there are many exceptions to the general rule. For example, apoptotic cells can be phagocytosed by dendritic cells (DCs), leading to presentation of antigens derived there-from on MHC class I molecules, a mechanism known as cross-presentation [6].

1.2. Modified immune responses in the ‘immune privileged’ brain

Certain observations of immune responses in the CNS have led to the designation of the brain parenchyma as an immune privileged site. This terminology arises from transplantation experiments in which there is extended survival of tissues transplanted to the CNS, compared with their survival in other sites [151,28]. In fact, rather than an aberration, it can be postulated that the risks associated with allowing a full-blown immune response to develop are too severe to permit in such a critical site as the brain. Uncontrolled inflammation would rapidly lead to a severe augmentation of intracranial pressure due to the volume limitations within the confines of the skull. This in turn would compromise neurologic function and augment death of critical cells that have rather limited capacity for renewal. To limit the possibilities of such a scenario, the brain is relatively well protected from physical injury by the thick skull and a degree of cushioning afforded by the CSF. Moreover, passive entry of many pathogenic organisms present in the blood is limited by the presence of the tight junctions present between the endothelial cells of the cerebral vasculature, the so-called blood–brain barrier (BBB) [41,198]. Apart from tight junctions with extremely high electrical resistance that limit paracellular diffusion, the BBB has many other special features not present in microvessels from other organs. The endothelial cells lack pinocytic vesicles that limit transcellular flux, they express distinct cytoplasmic enzymes, and they possess polarized specific transport systems for import of metabolites and export of toxic waste from the brain. Whilst the endothelial cell barrier is the ‘front-line’ of the BBB, its integrity is maintained by other cells that intimately contact its abluminal surface. Astrocytes are particularly implicated, they almost totally surround the vessel with their foot processes [119], but pericytes and perivascular cells may also be important. The overall consequence of the presence of the BBB is that access to the brain parenchyma is limited for many components of the immune system. Thus, concentrations of immunoglobulins and complement components are significantly lower than those found in plasma. Immune cell trafficking, whilst not
excluded, is more limited than for other sites [105]. Furthermore, a lack of organized lymphatic drainage and low MHC expression in the brain parenchyma are factors that contribute to the regulation of immune responses, but not for their abrogation. Such considerations should lead us to understand immune privilege in the CNS as a relative rather than an absolute term. In fact the choice of terminology to describe the nature of CNS immune responses should certainly not be fixed—in terms of detecting spontaneous immune responses, we are now more adept at assessing them than we used to be. And as far as immunization is concerned, we now understand much more about the basic rules of immune responses, and we can induce responses under conditions where this was not previously possible. Thus, we are currently at an exciting moment for neuroimmunoLOGY. Firstly we can incorporate many of the enormous conceptual advances in general immunology to better understand immune responses in the CNS. And secondly, we can hope to profit from recently developed procedures and technology to manipulate immune responses if we make appropriate modifications for the particularities of the brain.

An anatomical consideration must be made for the discussions that will be developed in this review. The ‘brain’ and the ‘CNS’ are clearly not a homogenous anatomical unit. The choroid plexus, the ventricles, the meninges and the CSF have more direct interactions with the systemic immune system than the brain parenchyma, arguably the ‘most privileged’ part of the CNS. One consequence is that in the former sites, immune responses are similar to those found elsewhere in the body, whereas the parenchyma may be considered to have rather subtle, low level interactions with the systemic immune system.

Immune responses are the result of complex interactions involving many cell types in specialized microenvironments. Nevertheless, many advances in our understanding of immunological concepts have evolved from somewhat reductionist studies on individual cell lineages and molecules. The discussions that follow are limited to T-cell immune responses, with a particular slant towards CD8+ T cells. This is principally due to the demonstrations in many animal models and now, in various tumor immunotherapies under clinical trial, that CD8+ T cells are the principal antitumor effector cell. Indeed, responses that are cell-mediated rather than humoral responses may be of greater significance for immune effector function in the intact CNS, because of the possibility of T cells to infiltrate brain parenchyma. As far as the focus on CD8+ T cells is concerned, these cells have the potential for the most exquisite fine specificity and a mechanism of action that is generally mediated by direct cell contact, without necessarily involving bystander killing of antigen-negative targets. Furthermore, for direct cell-mediated effector function, there are a significantly wider number of potential target cells in the brain that express MHC class I molecules than those that express MHC class II molecules.

1.3. Initiation of primary immune responses

There have been enormous advances over the past few years in our understanding of how T-cell immune responses are initiated in vivo. This necessitates subtle choreography involving several cell types and molecules, with interactions occurring at different anatomical sites. These fundamental aspects need to be discussed before the particularities of the CNS are considered.

The sooner that T lymphocytes are alerted to the presence of an antigen, the better the protection they can afford the host. Most antigens derived from pathogens or from newly formed malignancies will be present only locally at early stages. The challenge to the immune system is how to orchestrate an encounter between a rare specific T cell (calculated to be around 1/100 000 naive T cells for certain immune responses: [124]) and an appropriately presented antigen. Considering the T cells in the first instance, before stimulation by antigen (i.e. naive T cells) they are in constant recirculation between blood and secondary lymphoid organs (lymph nodes, tonsils, Peyer’s patches and the spleen). Naive T cells are not generally found in healthy non-lymphoid tissues and are essentially absent from normal brain, as detected by immunohistochemistry [102]. More recent observations have challenged this assumption, although the results are still consistent with a preferential trafficking of activated T cells through the CNS [39]. In the inflamed brain, there may be BBB disruption as a result of the relaxation of tight junctions and upregulation of adhesion molecules and chemokines [49,122]. This does not result in unrestricted leakage of blood cells into the brain (for example, erythrocytes are rarely found in the immune infiltrate). However, naive antigen inexperienced CD4+ T cells are detected in the brain parenchyma under these conditions [133]. The possibility that such naive T cells may be present in the brain emphasizes the importance of understanding antigen presentation in the CNS, but to date our understanding of naive T-cell activation is best understood in the secondary lymphoid organs, particularly the lymph nodes and Peyer’s patches, into which T cells enter via the high endothelial venules. These specialized vessels express adhesion molecules termed addressins that engage with T-selectin and integrin molecules expressed by naive T cells. It is within the specialized architecture of lymphoid organs that naive T cells have the opportunity to encounter their specific antigen on an APC (typically a DC). Encounters between naive T cells and mature DCs in the paracortical area are facilitated by chemokine gradients [97,165,48], and by a specialized extracellular matrix that encourages T-cell migration and serial sampling of DCs [98]. At this moment, there are two possible outcomes. If there is no encounter with specific antigen, T cells will leave via the efferent lymph to re-enter the blood at the thoracic duct. Alternatively, antigen specific activation occurs.

Naive T-cell activation has particularly stringent require-
ments. If only the minimum ligand for antigen recognition is detected, i.e. the correct peptide and MHC combination, this will induce signaling in naive T cells that results in anergy or apoptosis. A second co-stimulatory signal is required to elicit full effector function of the T cell. The best defined costimulation pathway is that mediated by CD80 and CD86 (B7.1 and B7.2) that interact with CD28 on T cells, however the same B7 molecules can also give rise to negative signals by binding to CTLA-4 [223]. More recently other costimulatory molecules and receptors have been identified, which may give different qualitative or quantitative outcomes to T-cell activation [1,111]. Based on in vitro and in vivo data, mature DCs appear to be ideally equipped to prime naive T cells (Fig. 1). They secrete chemokines (MIP-3β and 6Ckine) to attract naive T cells [48,66,206] which are then encouraged to form clusters with DCs through interactions between adhesion molecules—integrins (β1 and β2) and immunoglobulin superfamily members (CD2, CD50, CD54 and CD58) [29,101]. These interactions may occur in specialized membrane domains referred to as ‘rafts’, which lead to the formation of an ‘immunological synapse’ between the T cell and the APC [95]. DCs then provide strong antigen-specific stimulation by virtue of a high density of MHC-peptide complexes at their surface, 10- to 100-fold higher than on B cells and monocytes [114]. Mature DCs are equipped to deliver a second signal to T cells by expression of a range of co-stimulatory molecules, of which CD86 is probably the most important. However, OX40 ligand and 4-1BB ligand are also expressed, with the former being particularly important for CD4⁺ T cells and IL-4 secretion [81], whilst 4-1BB ligand may preferentially stimulate CD8⁺ T cells and interferon-γ secretion [130,222].

1.4. Antigen capture

Since the initial source of an antigen challenge will only rarely be directly in lymphoid organs, the transport and localization of these antigens must be considered. The spleen is a somewhat special case, since its architecture enables a direct sampling of blood-born antigens that can then be taken up directly by resident DCs. For other lymphoid tissue, antigenic material draining from tissues accumulates in the lymph and is transported to the lymph nodes via the afferent lymphatics. However the route that is now becoming more clearly defined is the active transportation of antigenic material by DCs [25]. Certain DCs are resident in normal tissues such as the Langerhans’ cells of the skin. Other DCs or their precursors may be recruited once an inflammatory response occurs. In their resting, or so-called ‘immature’ form, DCs continuously sample the local environment by endocytosing microorganisms, dead cells and cellular debris. However, knowing the recirculation patterns of naive T cells, the likelihood of them stimulating a naive T cell of the right specificity directly in peripheral tissue is rare. If the immature DCs receive signals that may be interpreted as ‘danger signals’, they undergo maturation, a series of events that triggers various phenotypic and functional changes that encourage migration via the afferent lymph to the T-cell areas of lymph nodes. Maturation signals

Fig. 1. Activation of naive T cells by dendritic cells. Antigen-specific activation of CD4⁺ or CD8⁺ T cells occurs optimally in secondary lymphoid tissue where naive T cells traffic and follow chemokine gradients to interact with dendritic cells. Efficient presentation of MHC class I/peptide complexes to CD8⁺ T cells or MHC class II/peptide complexes to CD4⁺ T cells is achieved by mature dendritic cells that express high levels of MHC molecules, adhesion molecules and costimulatory molecules.
include pathogen-associated molecular patterns on the surface of microorganisms, and endogenous factors such as interferon-γ released from virally infected cells and heat shock proteins liberated as a result of necrotic cell death. Maturation not only allows the DC to migrate to lymphoid organs, it also diminishes the phagocytic and endocytic capacities of the DC, whilst upregulating its costimulatory capacity. The final stages of DC activation are only completed after interaction with CD4+ T cells, in which activation signals work in both directions, with the CD4+ cell activating the DC via CD40–CD40L interactions as well as it becoming activated itself. The mature ‘conditioned’ DC is then in an appropriate activation state to prime CD8+ T cells [137,194,214,32].

1.5. Antigen capture in the brain and initiation of CNS immune responses

In the light of the usual recirculation patterns of naive T cells and the optimal microenvironment for T-cell priming in secondary lymphoid organs, it can be reasonably proposed that the initiation of T-cell immune responses to brain-derived antigens will occur in such sites. However, the highly particular cellular composition of the normal brain parenchyma requires consideration of hypotheses that take into account these features (Fig. 2).

1.5.1. Hypothesis A

Here, we can consider that T-cell priming will occur as in other sites, by uptake of antigen by immature DCs, then subsequent DC maturation and migration to lymph nodes. However, there is an absence of resident DCs in the normal brain parenchyma, but in an ongoing inflammatory response, DCs have been detected [234], although their maturation status was not assessed. If these cells derive from DC precursors, recruited only after substantial inflammatory responses have been initiated and sustained solely by innate immune mechanisms, it would be expected that T-cell responses would be significantly delayed. There is evidence for and against such kinetics. Delayed immune responses in the brain compared with other sites were reported in early transplant studies [151,28], although the details of the mechanism responsible for eventual graft rejection were not studied. In contrast, more recent studies with transplantable tumors [243,91,132] and neurotropic viruses [182,197] showed very rapid kinetics. However, with all studies reliant upon intracranial implantation of cells or viruses, the absolute integrity of normal brain structure cannot be totally guaranteed. Antigenic material may thus potentially leak to the periphery, bypassing normal CNS antigen handling mechanisms. The other unknown factor for recruitment of DC precursors such as monocytes to an inflamed brain is whether differentiation to an immature DC can occur in the brain parenchyma. Indeed, monocytes recruited to the brain at various stages of fetal and adult life are thought to undergo a unique differentiation program depending upon the degree of infiltration into the brain parenchyma, that results in the phenotype of a perivascular or parenchymal microglial cell. Indeed, the functional capacities of microglia residing deep in the parenchyma are restricted, with much gene expression turned off [181]. Whether alternative differentiation pathways exist in the CNS is unknown. However, in vitro studies of cells derived from new-born mouse brain and cultured with different cytokines including GM-CSF, M-CSF, and in some cases in co-culture with astrocytes, demonstrated that cells with certain similarities to DCs could be cultured [11,209,188], but only limited functional and phenotypic analyses were carried out. In vivo, DC like cells have been described in the brain parenchyma of mice suffering from experimental autoimmune encephalomyelitis (EAE) or from those parasitized by Toxoplasma gondii [234,80]. In the latter case, functional studies were also carried out in which brain-derived DCs efficiently triggered antigen-specific and primary allogeneic T-cell responses and secreted IL-12.

1.5.2. Hypothesis B

An alternative possibility for T-cell priming is that APCs do not transport antigen to lymphoid organs, but there is drainage of soluble or particulate antigenic material. This has been demonstrated in several studies, principally using radiolabeled materials (reviewed by Cserr and Knopf [57]). Antigens present in the brain parenchyma are predicted to drain with the flow of ISF. This is produced as a filtrate of plasma across the capillary endothelium, it bathes the cells of the brain parenchyma, picking up antigenic material, then finds the route of least resistance—probably the perivascular spaces, to eventually drain either into the subarachnoid space with the CSF, then to venous blood via arachnoid granulations, or to lymphatics via arachnoid sheaths of certain cranial nerves and spinal nerve roots to finally reach the cervical lymph nodes. In support of the proposed drainage patterns of potentially antigenic material with the ISF, high molecular weight radiolabeled substances infused into the brain parenchyma preferentially accumulate in the deep cervical lymph nodes. In contrast, lower molecular weight substances are lost to the blood, presumably due to less restricted passage through vascular endothelium. Similar experiments, with similar conclusions, were also carried out using transfer of whole cells (lymphocytes, erythrocytes and macrophages) in some early studies [57,169]. However, we currently understand cell migration to be very dependent on adhesion molecule expression and chemokine gradients and since many such factors have only recently been defined, these earlier studies are difficult to interpret. Functional data supporting the importance of the cervical lymph nodes in CNS responses have shown that their removal diminishes B- and T-cell immune responses to antigens expressed in the brain [183,100]. A cautionary remark must be made regarding generalizations about antigen
Fig. 2. Hypothetical mechanisms of T-cell immune response induction to primary cerebral malignancies. (A) Sampling of tumor-derived antigens by dendritic cells (DCs), recruited to the tumor site after initiation of an inflammatory response (see Sections 1.4 and 1.5). After phagocytosis of antigenic material, DCs may differentiate to a mature phenotype under the influence of local factors expressed by stressed cells, such as heat shock proteins. Mature DCs migrate to secondary lymphoid tissue via pathways that are still undefined for the brain, where they activate naive T cells to differentiate and undergo clonal expansion. (B) Tumor debris and antigens derived therefrom drain with the ISF and potentially the CSF (see Section 1.5) to reach secondary lymphoid tissue wherein antigenic material is phagocytosed by resident APCs. If these APCs are also induced to express costimulatory molecules, activation and clonal expansion of naive T cells can occur. In the absence of costimulation, tolerance may ensue. (C) Antigen capture by a brain resident APC such as a microglial cell (as opposed to a DC), followed by migration to secondary lymphoid tissue by a currently undefined pathway. Since both positive and negative outcomes of interactions with T cells have been reported for microglia (see Section 2.4), the consequences in the secondary lymphoid tissue may include tolerance induction as well as T-cell activation.
drainage pathways from the brain parenchyma. This concerns the models and techniques used. Injecting with precision into defined CNS sites in small laboratory animals is technically difficult. Techniques pioneered by Csern are able to limit BBB damage and involve the infusion of very small volumes in order to minimize technical artifact [100], but it is doubtful whether such precision is reproducibly obtained by all researchers in the field. Another consideration is that the degree of communication between ISF and CSF in different species may not be identical, with more separation between these compartments in man than in rats [247]. Overall, these data offer convincing evidence for a degree of antigen drainage from brain parenchyma, but certain difficulties remain. The first is that if parenchymal antigens follow similar drainage patterns to the CSF, why does antigen implanted directly in the CSF induce much better responses than that implanted in the parenchyma [132]? One explanation may be that this is a reflection of the quantity of fluids draining from the brain, with 90% being CSF and only 10% ISF in the rat [57]. The other consideration is that whilst protein and other antigens may be readily taken up by phagocytic APCs, these cells require signals (for example, mediated by pathogen expressed molecules or host expressed stress proteins) to induce maturation to a state where they can activate naive T cells. It is far from clear whether such stimuli will always accompany efflux of potentially antigenic material from the brain. The important consequence is that an immature DC that phagocytoses antigenic material draining from the brain may not adequately express costimulatory molecules and may thereby tolerize rather than activate naïve T cells.

1.5.3. Hypothesis C

A further mechanism by which T cells may be primed to brain-derived antigens is that the antigen is transported to the lymph node by a cell other than a DC. To date there is no direct evidence in support of this hypothesis, but there have been certain tantalizing observations. The first was in a transplant model, in which rat brain allografts were implanted into the brain parenchyma. A small number of macrophage like cells expressing donor MHC were found in host lymph node and spleen [42]. In a recent study [185] in a totally syngeneic system, 2 h after intracerebral injection of antigen there was co-localization of the antigen with Mac1+ cells (staining macrophages/microglial cells) in the brain parenchyma. After 4 h antigen+, Mac1+ cells were detectable in the cervical lymph node. However, this result would be compatible with drainage of either free antigen or APCs. Whilst of great interest, neither of these studies directly established APC-mediated transport of antigen from brain parenchyma to lymphoid organs and subsequent activation of naive T cells. It is clear that much technical ingenuity will be necessary to directly address such questions, particularly in small laboratory animals.

These hypotheses for initiation of immune responses in the CNS are not mutually exclusive, and each may each apply in different situations. ‘Classical’ immune response induction, by DC uptake of antigen (Hypothesis A) is most likely to occur in the context of acute inflammatory states, such as in infection or trauma induced either experimentally/surgically, or by injury. Efficient immune response induction in the absence of local APC involvement (Hypothesis B) is improbable unless there is a total absence of signals induced by stress or infection, thus this is likely to occur under experimental conditions in which antigenic substances are infused intracerebrally. We consider that the final hypothesis, invoking unique brain APCs (Hypothesis C) particularly merits further investigation, since it is the resident APCs that have the first opportunity for CNS antigen uptake. As we elucidate the behavior of such cells, it should be possible to determine whether this hypothesis is totally independent of a DC hypothesis, or whether local APCs readily differentiate into a DC-like cell, or whether DC precursors are efficiently recruited as a more efficient second line APC.

2. Effector stages of T-cell immune responses

Whilst the mechanisms responsible for the induction of spontaneous T-cell immune responses in the CNS are hypothetical, there are more direct data concerning the effector phase of the response. T cells infiltrating the brain parenchyma can be directly visualized in both clinical and experimental situations and in some cases, sufficient cells can be isolated for ex vivo functional tests.

2.1. Entry of primed T cells into the CNS

Both in vivo and in vitro data have contributed to our current understanding of how T cells pass the BBB to enter the brain parenchyma. The earliest published experiments detailing this T-cell traffic were those of Wekerle et al. [246], who showed that adoptively transferred T cells entered the brain, regardless of their specificity, as long as they were activated. Later studies by Hickey et al. [106] showed that after in vitro activation with mitogens, CD4+ and CD8+ T cells rapidly entered the brain parenchyma, with CNS concentration peaking at 9–12 h after intravenous transfer. CD4+ T cells reactive with a CNS expressed antigen (myelin basic protein) were selectively retained in the brain parenchyma, whereas cells of other specificities exited within 1 to 2 days. Similar findings were reported for CD8+ T cells specific for a viral antigen expressed in the brain [116]. Thus, immunosurveillance by activated T cells would appear to follow similar rules for the brain as for other sites [45], except that T-cell entry to other sites may be significantly more efficient. For example, in a rat model, adoptively transferred activated T cells entered the brain parenchyma much less efficiently than for other sites (six times less than in muscle and more than 140 times less
2.2. Molecules involved in transmigration

Lymphocyte transmigration across the BBB broadly proceeds according to the multistep model of homing [228,264]. Tethering is the first step, in which activated T cells make contact with endothelial cells principally with the tips of their microvillus surface protrusions, forming temporary, low affinity bonds that rapidly dissociate at the cell’s upstream end, but which are replaced by new bonds formed downstream. This binding is sufficient to reduce the velocity of the T cells to a rolling motion along the vessel wall. The bonds are principally between selectin molecules and oligosaccharides, with P-selectin on the endothelial cell being particularly implicated for the initial tethering of CD4+ T cells to non-inflamed brain vasculature [50], although this has been contested by other groups [239]. Higher affinity interactions are necessary to fully arrest cells to permit diapedesis into the extracellular matrix, these are mediated by integrins expressed by the T cell (e.g. LFA-1, α4β1, αβ7) interacting with cell adhesion molecules (CAMs) on the endothelial cell (e.g. ICAM-1, VCAM-1, MAdCAM-1). This interaction may require T-cell integrin activation by chemokines immobilized on endothelial cells interacting with receptors on T cells, transmitting signals through G proteins [239]. Signaling to the brain endothelial cell may also be important, particularly through ICAM-1. This has been proposed to stimulate a reorganization of the endothelial cell actin cytoskeleton, leading to either pore formation or disaggregation of tight junctions, thereby facilitating T lymphocyte diapedesis [3]. However, modifications of the multistep paradigm of transendothelial migration have been postulated for the CNS, in which α4-integrin may be able to mediate binding to VCAM-1 and possibly MAdCAM-1 under physiological flow (thus permitting tethering, although a rolling step may not be obligatory) as well as under static conditions [239,72]. The complexities and subtleties of T-cell/endothelial cell interactions are an active area of current research and undoubtedly involve many molecular interactions, not all of which have been fully characterized. Furthermore, the importance of different interactions will vary according to whether T cells are 'pioneer' T cells, entering a non-inflamed CNS, or whether an inflammatory response is under way [116,50,239,43,12]. Thus, early innate immune responses (which are very difficult to adequately model in EAE systems or after intracerebral implantation) will certainly influence the subsequent recruitment of specific T cells by the induction of adhesion molecule expression. In chronic conditions such as in the case of malignant astrocytoma, the tumor cells themselves can influence leukocyte infiltration by secretion of chemotactic factors or factors regulating adhesion molecule expression [61,213,240]. A challenge of future research in neuroimmunology is that current advances in ‘mainstream’ immunology in defining subsets of CD4+ T cells such as Th1 and Th2 [157] and regulatory populations [189], CD8+ T-cell Tc1 or Tc2 subsets [158] as well as populations of memory or effector subsets based on chemokine receptor expression [205,138] are investigated in the context of CNS immune responses.

2.3. Antigen-specific restimulation in the brain: are specialized APCs necessary for CD8+ as well as CD4+ T cells?

Upon T-cell entry into the perivascular space, T cells will be ready to pursue a variety of careers depending upon the stimuli they receive there. The minimum requirements for antigen recognition are MHC class I expression for CD8+ T cells and MHC class II expression for CD4+ T cells, together with the appropriate antigenic peptide. This may be adequate for eliciting certain biological functions (cytolytic activity, cytokine release) from an effector T cell with a high affinity TCR, but other factors such as expression of adhesion molecules, costimulatory molecules and cytokines can make antigen presentation more efficient. The question of brain APCs at the effector stages of immune responses is generally thought of as being particularly critical for CD4+ T cells, because MHC Class II expression is less widely expressed than MHC class I, even after exposure to inflammatory cytokines. Furthermore, CD4+ T cells are frequently not the ultimate effector cell in an immune response, they may be working through macrophages or B cells for example, which do not require MHC expression to exert their effector functions. Whilst this may occasionally be the case for CD8+ T cells [255], most fully differentiated classical CTLs are thought to directly kill any target expressing adequate peptide/MHC class I complexes at the surface, for example a virally infected cell or a tumor cell. The question therefore arises as to whether CD8+ T cells have any requirement for additional contacts with other APCs. In the case of antitumor immune responses we have recently demonstrated that tumor-specific, brain infiltrating CTLs do interact with brain APCs (Calzascia et al., manuscript in preparation). The functional consequences of antitumor CTLs interacting not only with their tumor cell targets, but also with local APCs have not yet been directly de-
termined, but data from different systems suggest that a variety of outcomes are possible (Fig. 3). Immune responses may be downregulated, either because of tolerogenic properties of the APC, or through the lysis of a stimulatory APC by a fully differentiated CTL. Alternatively, there could be an amplification of the immune response, for one or several of the following reasons.

Recruitment of CD8+ T cells from the periphery may be enhanced by the presence of APCs in the brain parenchyma [51]. Furthermore, the subsequent penetration of T

![Diagram](image)

Fig. 3. Effector stage of a T-cell-mediated response to a cerebral malignancy. T cells activated in the periphery extravasate through the intact or locally compromised blood–brain barrier (BBB). Once in the perivascular space, there will be encounters with APCs such as microglial cells and dendritic cells (DCs) (see Section 2.3). (A) Interactions with APCs expressing costimulatory molecules may maintain viability and function of effector T-cell populations, and counteract immunosuppression mediated by endogenous or tumor-derived factors such as TGF-β. Synergistic co-operation between T-cell subsets (CD4+ and CD8+) may also be encouraged through T-cell clustering around an APC expressing both MHC class I and class II molecules. Efficient antitumor effector function can thereby result. (B) Interactions with local APCs that regulate immune function in order to limit neuroinflammation may also restrict antitumor function. T cells may be tolerized or retained in peritumoral regions and those that infiltrate the tumor matrix may succumb to immunosuppression mediated by tumor expressed factors such as TGF-β and FasL, leading to tumor immune escape.
cells from the perivascular space into the parenchyma may be dependent upon interactions with perivascular APCs, although to date this has only been demonstrated for CD4+ T cells [235]. The presence of costimulatory molecules by the APC may protect CTLs from activation induced cell death and thus augment the magnitude of the response at the population level [112,140,37]. Moreover, the potent stimulatory capacity of a professional APC may enable CTLs to fully differentiate or overcome inhibitory microenvironmental factors such as TGF-β [91,92]. It is also possible that on the rare occasions when naive T cells can enter the CNS, after BBB breakdown or inflammation [133], a fully mature and activated professional APC would be able to activate naive T cells. The benefits of APCs for CD8+ T cells may also be indirect, with CTLs benefiting from cytokines (e.g. IL-2) released by CD4+ T cells clustered around a MHC class II+ APC, or other cytokines (e.g. IL-12) liberated by an APC stimulated by the CD4+ T cell. The consequence may be local microenvironmental conditions that protect against apoptosis and encourage a degree of intracerebral T-cell clonal expansion [161]. Priorities of future research investigating APCs for CD8+ T cells include the demonstration of local APC function in different models or neuropathologies. Thereafter, modulation of such functions can be most rationally attempted if the responsible cell type is defined (as discussed below).

2.4. APC candidates in the brain

Whilst few cell types present in the normal brain constitutively express MHC molecules, once an inflammatory response is under way many resident or recruited cells are MHC positive and thus may contribute to antigen presentation. There are many technical difficulties in isolating sufficient numbers of a given candidate brain APC population to perform ex vivo analyses. Many researchers in the field have thus resorted to cultured cells, often from fetal or newborn animals to assess function. Whilst these studies are informative, culture systems from different laboratories are rarely identical and none can totally match the complex interactions occurring in vivo. The in vitro properties of the various cell types thus defined are best interpreted as potential functions, which must eventually be confirmed in vivo.

Following extravasation of T cells, the first potential encounters with APCs will be with endothelial cells and capillary pericytes, on which MHC class II molecule and adhesion molecule expression is inducible by interferon-γ, tumor necrosis factor-α, and IL-1 [75,74,179,23]. Endothelial cells may even be in a position to present antigen to T cells at the luminal surface, however since T cells extravasate regardless of their antigen specificity [105], this is probably not of great physiological importance. Pericytes are an interesting APC candidate, since in some differentiation states they can acquire macrophage like functions such as phagocytosis and (in vitro) presentation of antigen to CD4+ T cells [105,10]. Such a process may result in local cytokine release, influencing endothelial cell phenotype and may thereby regulate lymphocyte extravasation.

Brain macrophages, generally referred to as microglial cells, are prime candidates for antigen presentation in the brain [209,10,8,9]. Although many subpopulations exist in different anatomical locations (meningeal macrophages, choroid plexus macrophages, ependymal cells), two broad categories will be considered here: parenchymal microglia and perivascular microglia [105,10]. Both populations are of hematopoietic origin, but their entry and turnover in the brain occurs at different times and rates. The parenchymal microglia populate the brain during fetal life and are long-lived cells, with a turnover of only a few percent per year [139]. Under non-pathological conditions, parenchymal microglia constitutively express few classical macrophage markers (or express them at low levels) and are poorly phagocytic. Perivascular microglia, bear more resemblance to conventional macrophages, they are a dynamic, constitutively phagocytic population that are regularly replenished, as originally observed in transplantation studies in rats, in which the grafting of bone marrow induced a new population of perivascular microglial cells of donor origin in the host [134,69]. When activated, microglial cells possess certain characteristics of professional APCs, with expression of CD1a, MHC class I and class II molecules, as well as adhesion and costimulatory molecules such as B7.1 (CD80), B7.2 (CD86), LFA3 (CD58), CD40 and ICAM-1 (CD54) [251,256,210]. In vitro, they can induce allogeneic T-cell responses and stimulate T-cell lines to secrete cytokines and proliferate. In vivo, B7 molecules are detected on reactive microglial cells present in inflammatory multiple sclerosis lesions [250] and in brains of mice suffering from Theiler’s virus-induced demyelinating disease [123]. Another in vivo observation was of donor T-cell-activated microglial cell clusters in graft-versus-host disease, in which the microglial cell strongly expressed CD11b/c and MHC class II [216]. Proliferation markers suggested that a proportion of the microglia were cycling. Some authors have suggested that microglial cells may have a role in tolerance induction, or the termination of immune responses [39,149,84,85]. Such effects were particularly associated with the activation state of the microglial cell [149], and with their parenchymal rather than perivascular localization [85].

A further non-professional APC candidate resident in the brain is the astrocyte. As far as normal astrocytes are concerned, MHC class I and II molecules are either undetectable or weakly expressed in vivo [136], whereas cultured astrocytes can express high level of MHC class I and II molecules, particularly after incubation with interferon-γ [63,245,148]. Cultured astrocytes can present foreign antigens to class I and class II restricted T cells
[63,245,226], but may be unable to trigger a complete T-cell activation program [8,245]. Furthermore, for presentation of antigens to CD4+ T cells, they are proposed to stimulate a Th2 T-cell immune response [1,10,8], rather than the Th1, pro-inflammatory response more readily elicited by activated microglial cells. Cultured astrocytes are considered to be the in vivo correlate of reactive astrocytes in vivo, readily induced after any form of brain trauma, possibly as a secondary consequence of microglial cell activation. However, in vivo induction of MHC expression in astrocytes is a controversial issue: certain studies suggest that MHC class I can be induced after viral infection or exposure to IFN-γ [55,221,120], whereas other authors have suggested that this will only occur if there is neuronal degeneration [109,190].

Evidence from different organs (e.g. liver [143]; eye [78]) indicates that local APC are key players in directing qualitative, site-specific aspects of immune responses. For the brain, many or all of the candidates discussed above may subtly influence local immune function. However, for immunomodulation in future clinical therapies, resident or recruited cells of hematopoietic origin, with professional APC function, are the most enticing candidates to target and manipulate.

3. T-cell immune responses to brain tumors in clinical and experimental situations

The principles of the induction of immune responses have been considered as well as how the effector phase of CNS responses may occur. The final outcome depends on the immune response that was induced and the efficiency of the effector phase of the response. In the case of tumors located in the CNS that will be considered in this section, simply observing whether a tumor is eliminated by an antitumor immune response is not always very enlightening. In order to understand whether and how immune responses directed towards brain tumors can operate, it is therefore necessary to look to systems in which it has been possible to observe and analyze immune parameters.

3.1. T-cell immune response against human malignant astrocytoma

Astrocytomas derive from neuroectodermal glial cells and are the most common primary brain neoplasms. The malignant forms include anaplastic astrocytoma (grade III) and glioblastoma (grade IV); these are tumors that progress rapidly and are almost invariably fatal. Indeed, despite advances in the application of combined modality cancer treatments [233], these have had little impact for malignant astrocytoma patients, with a median survival rate of less than 12 months for glioblastomas [232]. From the neuro-immunological standpoint, it is of interest that these tumors arise and remain in the CNS, they are highly infiltrative but do not generally metastasize outside the brain. It may be assumed that at the earliest stages of neoplastic transformation, the first tumor cells are totally contained within the normal architecture of the brain. Such primary brain tumors thus present a unique challenge for CNS immunosurveillance, since unlike viral, bacterial or parasitic infections, the systemic immune system will not have had the possibility of a direct contact with foreign or mutated self antigens. However, at later stages of tumor progression there may be some destruction of BBB integrity as well as substantial neoangiogenesis, resulting in intratumoral vessels that do not bear the hallmarks of the BBB.

Immunohistological and molecular analyses of human malignant astrocytoma have shown that T-cell infiltration is a frequent occurrence [196,230,180], but it has only occasionally been correlated with a favorable prognosis [44]. However, in the absence of a definition of the specificity and function of the infiltrating T cells, the significance of these findings is not clear. The first issue that must be addressed is whether malignant astrocytoma cells are sufficiently antigenically distinct from normal tissue to be recognized by tumor-specific T cells. The second is whether T cells activated by tumor expressed antigens are capable of retaining effector function when infiltrating a tumor growing in the brain parenchyma.

It is apparent from recent literature that newly defined antigens expressed by human tumors are regularly being characterized [244,191]. However, few results to date have been published concerning astrocytoma antigens able to spontaneously elicit an immune response. Most studies have assessed astrocytoma associated antigen expression at the mRNA level [54,204,212] or by serology [53], rather than T-cell defined antigens. Many of the antigens screened for in these studies are those that have been previously defined in melanoma, a reasonable starting point given the common neuroectodermal origin of melanocytes and astrocytes. The list of antigens potentially expressed by at least some astrocytomas includes several antigens that are not totally tumor-specific, but that have limited expression in normal somatic cells. The list of putative antigens includes MAGE and GAGE family members, tyrosinase, TRP-1, TRP-2, gp100, p97, SSX-1, SSX-2, SSX-4, SCP-1, and TS85. Expression of T-cell epitopes derived from these antigens at the tumor cell surface has been rarely studied. However, MAGE-1 antigen expression is detected in some cultured astrocytoma cells and can be recognized by specific CTL, but this antigen is not generally expressed by astrocytomas in vivo [212,58], probably because of a different level of DNA methylation induced by culture [59]. Further antigens that are recognized by CTL in vitro, are SART1259, originally identified in epithelial cancer cells, but now also shown to be expressed in malignant astrocytoma biopsies [113], and an epitope from the IL-13 receptor α2 chain [173,60]. The list of astrocytoma antigens confirmed to be capable of
stimulating T cells has remained limited for certain very practical reasons. The classical approach to detecting tumor-specific T cells is the co-culture of patients' T cells with putative antigen expressing autologous tumor cells, together with appropriate T-cell growth factors such as IL-2. This may allow preferential expansion of antigen specific T cells, which can subsequently be tested for specific, MHC-restricted recognition of the autologous tumor cell lines, or antigens derived therefrom. If the tumor infiltrating T cells are isolated, they may be preferentially enriched for tumor-specific cells, due to antigen-specific retention at the tumor site, as previously discussed. Astrocytoma infiltrating T lymphocytes can be isolated for culture, although the yield is generally modest due to the limited biopsy size and a degree of infiltration that is less intense than that found for certain other tumors such as melanoma. The relatively limited number of cells that can be isolated necessitates significant in vitro expansion to generate sufficient cells for testing. However, this problem is compounded by the fact that astrocytoma infiltrating lymphocytes (as for T cells in certain other diseases) exhibit reduced proliferative potential in culture and those cells that do grow in vitro show an important skewing during the culture period [65,154,159] and may not necessarily be representative of the starting population of tumor infiltrating lymphocytes.

To circumvent such limitations, we have adopted a novel molecular strategy to analyze α/β TCR+ T cells present in the immune infiltrate and in the blood of malignant astrocytoma patients. As previously discussed, α/β TCR+ T cells recognize antigenic peptides presented by MHC molecules using a heterodimer composed of an α and a β chain, the hypervariable complementarity determining region (CDR) 3 region of which carries the principal antigenic specificity of a T cell. We have used TCR spectratyping, a high resolution reverse-transcription polymerase chain reaction-based technique that determines TCR β chain CDR3 length (five to 15 amino acids) within the different V gene families. This is a powerful approach to study TCR diversity in a blood or tissue RNA sample, and thus to detect T-cell clonal expansions in vivo [150,176]. Indeed, the identification of recurrent CDR3 regions in large T-cell populations generally indicates antigen driven expansion of the corresponding T-cell clones. The analysis of a large panel of biopsies from malignant astrocytoma patients showed that oligoclonal expansions of T cells were present within various Vβ subfamilies of all biopsies tested [180]. In contrast to this local reaction, a systemic immune response was not generally detectable, since only exceptional oligoclonal expansions were detected in peripheral blood. It is therefore unlikely that T cells clonally expanded in astrocytoma are the direct consequence of blood expansions, such as those observed in certain healthy people [73,215,164]. These data for brain tumors are similar to those found for other types of cancers [147,77,220,47,46]. Overall, conservation of structural features of the critical TCR CDR3 region among the striking oligoclonal expansions that we observed in astrocytoma is most adequately explained as being the result of tumor antigen-driven clonal expansion of specific T cells. However, it is clear that the full significance of these cells will only be fully unraveled when their specificity can be determined.

The principal oligoclonal expansions of T cells with homogeneous CDR3 length were mainly CD8+ T cells, in the patients that we examined. However, judging from immunohistological and flow cytometric analysis, similar numbers of CD4+ and CD8+ T cells infiltrate the tumor. This obviously raises the question as to the function and specificity of the infiltrating CD4+ T cells. Are these inflammatory cells recruited to the tumor site, but not specific for any locally expressed antigen? Are they in fact specific, but with heterogeneous CDR3 expression? Is the number of CD4+ T-cell epitopes relatively large, so retaining CD4+ T cells of many specificities? Is the absence of dominant CD4+ T-cell oligoclonal expansions a reflection of an inadequate CD4 arm of the response, and thus a factor that may limit the efficacy of the CD8+ cells that co-infiltrate? These are important questions, but ones that we cannot answer without more knowledge about the specificity and function of the CD4+ cells. Indeed, our understanding of CD4+ T cells in tumor immunology is less advanced than for CD8+ T cells, there are fewer MHC class II restricted tumor antigens that have been defined and the characteristics of the CD4+ T-cell repertoire specific for these antigens are unknown.

3.2. Immune escape of astrocytomas

If optimal stimulation of the immune system does not occur when the tumor mass is small, elimination of a solid brain tumor by immune effector cells is a formidable challenge. Part of the problem may be the magnitude of the immune response that is induced: the immune infiltration that is induced may be insufficient. But the tumor cells may also resist immunological eradication by various passive and active mechanisms of immune escape. Indeed, over the past three decades, many defects have been reported in the functional status of T cells in brain tumor patients, concerning circulating T cells as well as those infiltrating the tumor [67]. In vivo manifestations of these defects include delayed type hypersensitivity responses, low T-cell numbers in peripheral blood and low serum antibody titers. In vitro tests indicated poor responses to mitogens, poor cytotoxic function, defective expression of high affinity IL-2 receptors and activation signaling defects [156]. Such phenomena may explain difficulties in culturing CTLs derived from astrocytoma infiltrating lymphocytes, despite the addition of recombinant IL-2 [71,155]. However, the real implications of these features cannot be fully assessed until it is known whether astrocytoma specific T-cell activity has been impaired. Nevertheless, in
the absence of a direct confirmation of this, many potentially immunosuppressive soluble or cell associated molecules have been characterized.

3.2.1. Immunosuppression by soluble factors
A list of potentially immunosuppressive molecules detected in astrocytomas or astrocytoma lines includes prostaglandin E₂ [67,56,83,211], gangliosides [249] and IL-10 [167,153,107], all of which can demonstrate certain immunosuppressive functions in vitro, but for which in vivo immunosuppression in astrocytoma patients remains speculative. However, one astrocytoma-derived factor that has been explored in more depth, in vitro and in vivo, is TGF-β. Indeed, this cytokine was originally called glioblastoma cell-derived T-cell suppressor factor and was first identified in the supernatant of a human glioblastoma cell line that suppressed T-cell growth [56,82,35]. The immunosuppressive effects of TGF-β are multiple and complex. They include the inhibition of maturation and antigen presentation by DCs or other APCs, inhibition of T-cell activation and differentiation towards effector cells (either cytotoxic cells expressing perforin or Th1 or Th2 cells) [224,115,93,117]. Inhibiting TGF-β in vivo in experimental tumor models has produced mixed results, probably because the effects of this cytokine are not only on the immune system, but also on the tumor cell, which may be protected from Fas (CD95)-mediated apoptosis in some circumstances [76,21]. Other in vivo studies using decorin, a natural inhibitor of TGF-β, suppressed the growth of C6 rat astrocytoma in vivo [229], but whether this occurred through TGF-β is unclear, because decorin is also immunostimulatory in a TGF-β2 independent fashion [160]. Overall, the immunoregulatory functions of TGF-β2 warrant the attention it has received, but whether it will be feasible or advisable to inhibit this cytokine in brain tumor patients still remains uncertain.

3.2.2. Immunosuppression by cell-mediated interactions
The situation for cell-mediated immunosuppressive factors is similar to that found for soluble factors, with several candidate molecules, but not all that have been thoroughly investigated in vivo. We investigated the possibility that glioma may utilize membrane-bound Fas ligand (FasL, CD95L) as a mechanism of immune escape. FasL belongs to the tumor necrosis factor family and is implicated in several biological functions through its interaction with Fas (CD95), a member of the tumor necrosis factor receptor/nerve growth factor receptor family. FasL–Fas interaction induces the trimerization of Fas and a subsequent complex cascade of intracellular events, potentially leading to apoptosis of Fas-positive cells, a mechanism central to immune homeostasis [162,146]. Indeed FasL⁺ astrocytoma cells (cell lines and also astrocytoma cells tested ex vivo) can specifically and efficiently kill Fas-transfected P815 target cells, but not wild-type P815 [202]. Moreover, we have also shown that a human astrocytoma cell line can kill CD4⁺ and also CD8⁺ T-cell lines derived from the autologous tumor, specifically using the FasL–Fas pathway [241]. Other groups have independently confirmed expression of FasL by astrocytoma [96,79,86] and, moreover, apoptotic T cells were observed in the proximity of FasL expressing astrocytoma cells in vivo [64]. However, other than this indirect in vivo evidence of a role for astrocytoma expression of FasL, the in vivo consequence of FasL expression by tumors has been a very controversial issue. Indeed, tumor expression of FasL in murine models has been correlated either with enhanced tumor growth [99,16], or with enhanced tumor rejection [218,219,17], with augmented neutrophil recruitment as a likely mechanism. Microenvironmental factors will certainly influence the consequences of FasL expression by tumor cells, for example, tumors co-expressing both FasL and TGF-β may be particularly well adapted to combat CTL effector mechanisms (discussed in Ref. [241]), a principle subsequently confirmed in in vivo models in which a FasL-positive colon carcinoma could escape rejection if TGF-β was also present [52]. These data help to explain the role of FasL in this particular model (with possible analogy to the situation in the brain), but it is likely that individual combinations of factors relevant to different tumors or models are responsible for the diverse interpretations of these issues in the literature [241,242,193,168].

Recently, HLA-G has been proposed as an astrocytoma expressed molecule with immunosuppressive potential [248]. This non-classical MHC class I molecule is expressed by a limited range of tissues, particularly the placenta, but also certain cancers. It is proposed to suppress NK and T-cell immune responses, but this is controversial [40,22]. Regarding astrocytomas, a proportion of astrocytoma cell lines and tumor biopsies expressed HLA-G protein and inhibition of CD4⁺ and CD8⁺ T-cell responses was demonstrated in vitro, but this was only tested after incubation of cell lines with high concentrations of IFN-γ (500 U/ml), or after gene transfer of HLA-G into glioma lines. A final candidate immunosuppressive molecule is the CD70 transmembrane glycoprotein, with expression by astrocytoma cell lines or in vivo now confirmed in two independent studies [252,104]. This molecule is also expressed on activated T and B cells, with roles in regulating immune responses via interaction with CD27, expressed on lymphoid cells [118]. In vitro functional tests suggested a pro-apoptotic role of tumor expressed CD70 when tested on PBMC targets; this was augmented (correlating with expression levels of CD70) when tumor cells were irradiated [252].

3.3. Immunotherapy—from rodent models to the clinic
In view of the limitations in analyzing immune responses to spontaneous tumors arising in humans, it may be hoped that appropriate experimental models will be
responses leading to efficacious antitumor activity are leukapheresis-derived autologous peripheral blood lympho-

tumor growth, expression of multiple mechanisms of immune escape. Since such a model does not exist, we may have to exploit several different systems for their applicability to model certain aspects of the tumor–host relationship. For the future, genetic models of ‘spontaneous’ brain tumors may be informative; indeed, these models incorporate some of the genetic features and heterogeneity typical of spontaneous human cancer [108]. However, such models have generally been designed to address genetic and pathologic issues and present a formidable challenge for immunological analyses. For the moment, models used to investigate immune responses against cerebral malignancies have relied upon transplantable tumors, generally implanted into defined regions of the brain under stereotaxic guidance. Overall, such models have provided encouragement for future immunotherapy, in that they have demonstrated that immune responses induced in the periphery can mediate antitumor effects in the brain [15,18,192,207,20,171,94,4,142]. However, optimization of any potential immunotherapy requires an assessment of the immune (or any other) mechanisms responsible for tumor rejection. An adequate characterization of immune-mediated rejection cannot really be achieved without knowledge of the antigenic targets of the antitumor response. Unfortunately, in certain rodent brain tumor models, characterization is sketchy and the systems are not always totally syngeneic [91,236,33], although some recent studies have used better characterized tumor models in the brains of syngeneic mice, such as B16/F10 melanoma [207,20], C3 sarcoma cells transfected with the human papilloma virus type 16 [171] and P815 cells transfected with the CW3 antigen [243].

With the findings that under ideal conditions in experimental models, vigorous CTL-mediated immune rejection of a cerebral malignancy can be achieved, can such results be directly translated to the clinic? Perhaps the principal limiting factor to date is the lack of defined tumor associated antigens that are expressed by a sufficient proportion of tumor cells in vivo to serve as tumor rejection antigens. Immunotherapy approaches for tumors in sites other than those of the CNS, in particular melanoma, have induced CTLs that react with differentiation antigens such as Melan-A [191]. Of course, such approaches may induce a degree of destruction of normal tissue [177], which is considered acceptable up to a certain point, although the balance of these autoimmune effects compared with antitumor effects is under scrutiny [262,26,68]. For immunotherapy of brain tumors, the threshold of acceptability of autoimmune reactions is lower because of the indispensability of most normal tissue in the brain and its limited capacity for self renewal. The related problem is that of inflammation. High magnitude immune responses leading to efficacious antitumor activity are frequently inflammatory. Furthermore, chronic inflammation may provoke a release of sequestered autoantigens, leading to the initiation or perpetuation of autoimmune disease. An indication of the possible risks of utilization of non-defined brain tumor vaccines came from early studies employing human glioma tissue to vaccinate different species of experimental animals: this resulted in lethal allergic encephalomyelitis [34]. Other unexpected untoward effects arose in rodent trials of a gene therapy protocol for glioblastoma, in which there was a vector induced intracerebral immune response that directly or indirectly perpetuated reactive gliosis and demyelination [62]. However, even immunotherapeutic approaches that appear to be well defined can give unexpected results that risk being uninterpretable if there is no possibility of a specific read-out for the antitumor immune responses. This was illustrated in a model in which tumor rejection of subcutaneous tumors was targeted towards a model antigen expressed by gene transfer in rat 9L glioma cells. However, the ultimate rejection of intracerebral tumor was mediated by immune effector cells of other specificities [142], that had presumably been induced by a process of epitope spreading, as seen in certain autoimmune diseases [135].

The somewhat overwhelming list of potential problems that may be encountered in any immunotherapy for brain tumors has not totally blocked the interest and implementation of clinical trials. This is understandable from a clinical perspective, given the lack of treatment options for patients with malignant astrocytoma. It is also relevant for researchers involved with more fundamental aspects of the CNS antitumor immune response, in order to stimulate and guide the creation of better models. An overview of the categories of immunotherapy which may be considered for treatment of brain tumors in a clinical setting is provided in Table 1. To date, an objective analysis of the published data from clinical trials is extremely limited due to variable descriptions of patient groups or disease status, as well as the sometimes premature reporting of survival data. With these limitations in mind, and with the absence of any obvious clinical breakthrough, immunotherapies will not be reviewed exhaustively, but rather for their future interest and potential.

3.3.1. Adoptive immunotherapy

This approach offers the advantage of circumventing deficits of antitumor effector cell populations in the host by providing optimal conditions for the culture and amplification of these cells in vitro, in the absence of tumor-derived immunosuppressive factors.

Most adoptive immunotherapy in glioma treatment to date has been a non-specific therapy, exploiting the cytotoxic properties of lymphokine activated killer (LAK) cells. Obtaining such cells is relatively simple compared with other cellular therapies. It involves the culture of leukapheresis-derived autologous peripheral blood lympho-
cytes with high concentrations of IL-2, this generates a polyclonal population of cytotoxic cells from cells of T and natural killer (NK) origin that are able to lyse NK-resistant targets, including tumor cells. However, the key characteristic of LAK activity against autologous tumor cells was not assessed in most trials. Strategies to maximize the potential cytotoxic function of LAK cells include the concomitant administration of IL-2 and the direct transfer to the tumor cavity at the time of resection (for a more detailed review of these therapeutic approaches, see Refs. [263,260]). Although this approach to experimental immunotherapy of brain tumors is one of the longest established and has yielded occasional isolated clinical responses, it is still not possible to confirm any clinical efficacy. Furthermore, there have been variable levels of toxicity associated with LAK cell and IL-2 therapy [27], mainly as a result of the cerebral edema induced by the treatment. When the critical and delicate nature of the CNS is taken into account, it is difficult to envisage how the therapeutic potential of a non-specific therapy reliant upon cytokines triggering intense inflammatory responses can be safely applied to brain tumors.

More sophisticated forms of adoptive immunotherapy that exploit the specificity of the immune system have been explored. As we have discussed in Section 3.1, tumor infiltrating lymphocytes should be enriched for T cells with classical, MHC restricted specificity for tumor cells. A pilot study using expanded tumor infiltrating lymphocytes has been undertaken, but with no evidence that the transferred cells were specifically cytotoxic for the autologous tumor [186]. The size of tumor biopsies available from CNS malignancies is extremely restricted and the few T cells derived from the tumor are difficult to expand in vitro, these factors will inevitably limit any widespread application of this approach. An interesting variant of adoptive immunotherapy is to attempt to increase the numbers of T cells used for in vitro culture by a prior vaccination of the patient with autologous tumor or tumor-derived antigens [254,184]. Since the patients are vaccinated at a site distant from the tumor, this may avoid the impact of tumor associated immunosuppression. However, whether any of the isolated clinical responses that were reported correlated with tumor specific effector function cannot be assessed, due to absent or inadequate characterization. Since even minor variations in vaccination procedures and subsequent in vitro T-cell culture significantly influence the function and quantity of antitumor effector cells, it is disappointing that we do not have the information to rationally propose improvements to these techniques. There have been recent developments in immunotherapy for tumors in sites other than the CNS that aim to ameliorate engraftment and antitumor effect of transferred cells by prior lymphodepletion of patients [68]. This approach shows certain promise for the therapy of melanoma, which if confirmed, may be possible to adapt for the treatment of malignant astrocytoma.

### 3.3.2. Active vaccination

Cancer vaccines fall into two broad categories: whole tumor cell/tumor cell-derived vaccines and defined antigen vaccines. Non-defined or whole cell vaccines require no prior definition of tumor antigens or MHC restriction elements, many epitopes are presumably present in the vaccine, lessening the considerable risk that tumor cells may mutate and escape a too finely focused immune response. However, assessing vaccine efficacy and correlating any clinical responses with specific immune responses is extremely difficult. Furthermore, as vaccine efficacy improves, there is a risk that autoimmune responses to normal tissue antigens present in the vaccine preparations may also be induced [34,144]. As previously argued elsewhere [170], rational improvement in non-defined vaccines is difficult once such approaches are incorporated into clinical trials, even if there is the hope of

### Table 1

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<thead>
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<th>Approach</th>
<th>Advantages</th>
<th>Drawbacks</th>
<th>Monitoring</th>
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<tr>
<td>Non-specific adoptive immunotherapy. LAK cells with high-dose IL-2</td>
<td>Efficient in vitro culture in the absence of local immunosuppression</td>
<td>Lack of specificity; inflammation</td>
<td>Main readout is clinical response</td>
</tr>
<tr>
<td>Specific adoptive immunotherapy</td>
<td>In vitro culture in the absence of local immunosuppression; tumor specificity can be measured before transfer</td>
<td>Sufficient in vivo sensitized cells difficult to obtain</td>
<td>Transferred cells can be tracked</td>
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<tr>
<td>Pre-defined vaccines. Whole tumor cell homogenate/eluate/RNA etc., with cytokine or DC adjuvants</td>
<td>Identification of tumor antigens not necessary; applicable to all HLA-types; broad immune response, limiting risk of tumor immune escape</td>
<td>Quantity of antigenic material needed to prepare vaccine; autoimmune; patient-specific vaccine preparation if autologous cells used; vaccine composition variable</td>
<td>Readouts based on 'whole cells' require autologous cellular controls —difficult for CNS</td>
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<tr>
<td>Defined vaccines. Peptides corresponding to CTL epitopes with synthetic adjuvants, or pulsed onto DCs</td>
<td>Standardized preparation for a given HLA-type; autologous tumor cells not needed; degree of expression on tumor and normal cells can be assessed</td>
<td>Lack of defined antigens for human astrocytoma; specific for individual HLA types; immune selection of tumor escape mutants</td>
<td>All tests for specific T-cell function possible, even without autologous control cells</td>
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benefit for isolated patients. The alternative strategy of inducing immune responses to defined antigens is more straightforward to interpret, but this requires that clinical trials fully integrate the collection of biological as well as clinical data. The hurdle for defined vaccines for brain tumors such as malignant astrocytoma is the paucity of antigen candidates able to be recognized by T cells (as discussed in Section 3.1). Therefore, to date, clinical trials have employed non-defined tumor-derived material for vaccination.

To induce immune responses against antigenic but poorly immunogenic human tumors requires efficient adjuvants that enhance and stimulate antigen presentation. This can be achieved either by exploiting existing host antigen presentation pathways in vivo, or by implanting ex vivo generated DCs, or DC/tumor cell hybrids as cellular adjuvants. The most common route to ameliorate endogenous DC function is to use recombinant cytokines, either secreted by gene transfer into tumor cells, or by the co-injection of a bystander secretor cell (reviewed in Ref. [178]). Key factors that have received much attention in this context are GM-CSF, IL-4, FLT3 ligand and CD40 ligand, which may find future application in brain tumor immunotherapy, and certain of which are already being exploited in clinical trials [172].

The ex vivo approach, of injecting cultured DCs, is of considerable interest because of the possibility of exactly controlling the parameters of the DCs that are injected. Recent advances in understanding how DCs play a central role in initiating, amplifying and regulating adaptive immune responses have provided much data on the ideal characteristic of an immunostimulatory DC [24]. The generalized method is to generate DCs ex vivo from bone marrow or peripheral blood precursors, load the DCs with tumor-derived antigens, then reinfuse into the animal or patient. Despite widespread application of these approaches, the technology is difficult to standardize and variation in antigen loading or the differentiation of cultured DCs used for injection can not only alter the magnitude of immune responses that are subsequently induced, but can also switch the balance towards tolerance induction [231]. Correlation of clinical efficacy with vaccine formulation can therefore only be adequately made if DCs used for treatment are adequately characterized, which, to date, has not always occurred.

The induction phase of antitumour vaccination in a peripheral site will be similar for most extracranial as well as intracranial tumors. Therefore, the design of DC-based brain tumor therapies can profit from the wealth of biological and clinical expertise now accumulating from other DC enhanced vaccines [231]. Indeed, the adjuvanticity of DCs has been confirmed in various brain tumor models [14,103,129,253,257,258,141,5]. Study design is so totally different in these reports that meaningful comparisons of efficacy are difficult to make, but many of the details of the outcomes have been previously reviewed [252]. Critical differences in the experiments include the species and strain of animal and corresponding tumor (rat and mouse), the use of DCs of different origin (DC cell lines, or spleen or bone marrow-derived precursors), the use of different sources of antigen (from RNA to whole cells) and different means of DC maturation. Finally, DCs were injected using different routes and frequencies, and at different stages relative to tumor implantation. Certain encouraging conclusions from these animal studies can nevertheless be drawn, namely that no severe side effects were encountered, and that there is some impact of immune effector cells on intracranial tumors. Treatment of malignant astrocytoma patients with DC-based therapies is now under way, and published results to date confirm that vaccines consisting of DCs pulsed with peptides eluted from tumor cells [259], and DCs fused with tumor cells [128], did not induce serious adverse effects. Evidently, efficacy can only be addressed in future randomized trials, but if a higher priority is given to obtain adequate immunological data, the future development of this otherwise exciting approach can be accelerated.

3.3.3. Immunomodulation with cytokines

Experimental therapy of brain tumors has also been attempted by employing cytokines that may act at the effector phase of the response. Indeed, there is a vast palette of recombinant cytokines available that can be employed by direct injection, gene therapy or via the use of transduced secretor cells. Certain preclinical studies have suggested that CTL expansion and function against brain tumors may be enhanced by IL-2 [90,127], IL-7 [15], IL-12 [127,121] and IL-18 [126]. Effector functions not exclusively mediated by CTLs may also be augmented by IL-4 [30,89] and IL-10 [38,217]. The challenge is to know which of these reagents merit development for clinical trial. Most of these cytokines have been tested in studies using totally different tumors and experimental protocols, at different doses, rendering comparisons of individual factors difficult to make. Assessing the potential interest of a particular cytokine is particularly acute for brain tumor therapy because there is no predominant model, unlike the situation for melanoma where preclinical testing of immunotherapies frequently employs the poorly immunogenic B16 melanoma. Nevertheless, it should be acknowledged that certain groups have published useful data comparing multiple therapeutic approaches in the same experimental brain tumor models [207,208,145]. The current limitation for advancing brain tumor immunotherapy is not the number of immunostimulatory molecules that are available, but rather our comprehension of the immune consequences (rather than just immune rejection) in the CNS. For example, cytokines employed locally that induce excessive inflammation may be less applicable to intracranial than extracranial sites. Or cytokines that interact with other cells that are unique to the CNS and that also participate in the immune dialogue, such as microglial...
cells and astrocytes [7,201,200,199], should be thoroughly investigated for their positive or negative regulatory effects. Certain new approaches to immunotherapy that are tailored for the CNS are particularly worthy of mention. One of the most interesting is based on the propensity of neural stem cells to track migrating glioma cells within the brain [2]. It was proposed that genetically manipulated neural stem cells could deliver therapeutic molecules with immunomodulatory function to the site of an infiltrative tumor. To date, encouraging results have been obtained with the delivery of IL-4 and IL-12 to experimental gliomas in rodents [31,70], but other molecules could also be exploited using the same approach.

3.3.4. Compatibility and synergy of antitumor immune responses with other therapies

Efficacious treatment of highly malignant brain tumors is unlikely to be achieved by single modality therapies. It is therefore useful to consider the compatibility and synergy of other therapies for CNS neoplastic disease with immunotherapy. Whilst chemotherapy and radiotherapy are poorly efficacious for malignant astrocytoma [233,110], they do induce a degree of tumor cell death and thus may provide a potentially immunostimulatory in vivo source of tumor antigen, provided the immune system has not been impaired by these treatments. Future protocol design should thus look to ways of appropriate staging of the different treatment elements to improve compatibility. Immune responses in gene therapies are a complex issue. On the positive side, they may be at least partly responsible for so-called ‘bystander effects’, of gene therapy, whereby a greater number of tumor cells are eliminated than are actually transduced by the therapeutic gene [163,238]. However, immune responses directed towards viral vectors, particularly early generation adenovirus vectors, limit repeat utilization of the vector and may also induce chronic neuroinflammation [62]. A particularly promising compatibility may be found between antiangiogenic therapies and immunotherapy [125,187]. Indeed, several of the cytokines already discussed as immunostimulatory agents (including interferon-γ, IL-4 and IL-12) may also directly or indirectly inhibit tumor angiogenesis.

Other therapies for brain tumors aim to exploit apoptosis pathways by engaging death receptors in malignant cells [36]. Fas ligand was one of the first molecules to be proposed and gave some encouraging results in an experimental gene therapy of rodent glioma [13]. However, Fas is expressed by so many different cell types, including cells of the immune system [162], normal astrocytes [200] and hepatocytes [195], that toxicity and immunosuppression may limit its clinical application. Even the effects of local administration of Fas ligand within the CNS are difficult to predict because of the potential of this molecule to manifest either pro- or anti-inflammatory effects at different stages of ongoing immune and inflammatory responses [174,261,203]. A more promising and less toxic therapeutic molecule is TNF-related apoptosis-inducing ligand (TRAIL) [19]. Although certain reports suggested some reactivity with normal cells of the brain [166], recent developments have indicated routes by which its therapeutic potential may be maximized by restoring apoptotic pathways in glioma cells using peptide drugs [87]. Using such an approach, apoptosis and tumor regression were achieved in an intracranial human xenografted glioma model after TRAIL administration [87].

4. Concluding remarks

There is now compelling evidence that CD8⁺ T-cell immune responses occur in the CNS and that at least in experimental models, CD8⁺ T cells can mediate protective roles such as the elimination of tumor cells. However, spontaneous antitumor immune responses in humans are inefficient, yet the need for new therapeutic approaches in brain tumors such as malignant astrocytoma is particularly urgent. We thus need to understand how antitumor responses can be safely amplified, taking into account the fact that the relationship between CNS malignancies and the immune system is likely to proceed along different lines than in other sites. Indeed, the tumor-host relationship is complex: many factors influence the final outcome of tumor growth, arrest or rejection, with the growth of aggressive brain tumors indicating that the balance is in favor of the tumor. We can speculate that this is a result of a series of circumstances that are prominent in the development of human astrocytoma, but which are absent in many experimental tumors.

Spontaneous human tumors inefficiently prime the immune system, resulting in a late response which is better adapted to minimize inflammation rather than to stimulate potent CTL activity. Furthermore, at early stages of tumor growth, there is little signaling of ‘danger’ until the tumor is well advanced. This is in contrast to certain experimental models in which the immune system may be alerted by the trauma associated with the surgical implantation of tumor cells.

The microenvironment of a large tumor mass that initially develops with little immune hindrance may become predominantly immunosuppressive. The low-level antitumor response that is eventually induced is then unable to mediate efficient effector function in the hostile tumor microenvironment in the brain. In contrast, the rapid response induced against certain experimental tumors (which in most cases do not liberate high levels of immunosuppressive factors such as TGF-β) is able to eliminate such tumors at an earlier stage, when they present a more manageable target for CTL attack.

The putative tumor antigens present in human astrocytoma may be expressed only at a low level, or they may be inefficiently processed or presented on MHC class I molecules. Furthermore, since many of the tumor an-
tigens may be overexpressed antigens also present on normal tissues, the host T cells may be tolerant, or of low avidity. Future studies with model brain tumor antigens in which there is also some expression on normal host cells will be of great interest in mimicking this clinical reality.

It may not be necessary to rectify all of the inadequacies of the spontaneous immune response to malignant brain tumors to achieve some clinical benefit. However, with an understanding of the basic rules for T-cell immune responses in the brain, rational proposals can be made to identify key areas in which to modify the neuroimmunological balance. This should be undertaken cautiously, since the particularities of spontaneous immune responses in the CNS have presumably evolved to minimize the risks associated with high level inflammatory immune responses. Successful immunotherapy for cerebral malignancies must find this narrow path between immune-mediated protection and immune-mediated pathology.

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