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Peripheral immune status in the GFAP-V^{12} HA-*ras* B8 spontaneous astrocytoma model

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“The real voyage of discovery consists

not in seeking new landscapes,

but in having new eyes.”

(M. Proust)

Alla mia famiglia,

per avermi sostenuto ed aiutato durante questo cammino così impegnativo...

per avermi spinto a fare solo quello che più mi piaceva e per non avermi mai posto alcun limite...

per avermi insegnato a non arrendermi e a sorridere in ogni situazione...

per tutta la stima e l'illimitata fiducia che sono sempre state riposte nelle mie mani...

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ABSTRACT

Glioma represents one of the deadliest primary CNS tumours. We used a transgenic spontaneous astrocytoma mouse model to analyse glioma-immune system interactions at the peripheral level. Importantly, this model allows analyses at early (asymptomatic) stages of the disease without the influence of concomitant treatments. In patients, local immunosuppression is induced by soluble factors and cell-associated molecules; in the periphery, T-cell hyporesponsiveness to *in vitro* mitogen-stimulation and a decreased CD4/CD8 T cell ratio were demonstrated. We performed functional and quantitative analyses on mouse LN and spleen cells to determine whether mice exhibit immunosuppression. In contrast to a parallel study of local immune status, there was little alteration in immune function or phenotype at early stages of tumour development. However, certain trends were apparent in symptomatic mice, and the CD4/CD8 ratio was significantly reduced. Furthermore, we used a recombinant virus vaccination to elicit or enhance an antigen-specific immune response. Although there was no significant difference between groups, a modest trend to have greater response in transgenic rather than control mice was observed. Overall, differently to local immune status, limited peripheral immune defects were restricted to later disease stages. Moreover, there were no significant alterations in specific immune response in transgenic mice.

RIASSUNTO

Glioma. Il glioma, tumore maligno alle cellule gliali, è uno tra i più comuni e mortali tumori primari al cervello. Anche a seguito di intervento chirurgico e chemio-radioterapia, la prognosi rimane limitata a meno di due anni. Esistono quattro differenti tipologie di glioma, secondo il tipo cellulare predominante di cui esso è composto: astrocitoma, ependimoma, oligodendroglioma e oligoastrocitoma. L'origine del glioma rimane ancora incerta; due meccanismi sono stati proposti per spiegare l'origine del tumore: la trasformazione di cellule staminali endogene e il de-differenziamento di astrociti maturi. A livello istologico, il glioma è caratterizzato da pleomorfismo nucleare, alta densità cellulare, figure mitotiche, necrosi e proliferazione dell'endotelio vascolare. Un aspetto peculiare di questo tipo di tumore è l'eterogeneità. Esso è inoltre molto invasivo e risulta costituito da cellule necrotiche circondate da una massa ad elevata attività angiogenica. A differenza di altri tumori solidi, il glioma non metastatizza al di fuori del sistema nervoso centrale. Esso viene classificato in quattro gradi, secondo la gravità crescente della patologia (classificazione dell'Organizzazione Mondiale della Sanità, 2007): I grado, caratterizzato da basso potenziale proliferativo e di possibile cura tramite resezione chirurgica; II grado, ricorrente e anch'esso interessato da basso potenziale proliferativo; III grado, tumore maligno contraddistinto da atipia nucleare; IV grado, definito glioblastoma multiforme, associato ad alta attività mitotica, elevata malignità e prognosi infausta. Vengono distinte due tipologie di glioblastoma multiforme: glioblastoma primario, il quale si sviluppa *de novo* in un periodo mediamente inferiore ai tre mesi, e secondario, il quale è la conseguenza di una lenta progressione da glioma di II o di III grado. Differenti mutazioni genetiche caratterizzano i due tipi di glioblastoma multiforme, supportando l'ipotesi che diversi meccanismi molecolari portano allo sviluppo di essi. Tuttavia, caratteristiche istologiche e risposta al trattamento sono comparabili tra i due.

Immunologia tumorale. In questo lavoro sperimentale, è stata in particolare studiata l'interazione tra tumore e sistema immunitario. Essa è stata recentemente designata dall'ipotesi dell'*immunoediting*, processo suddiviso in tre fasi temporaneamente conseguenti: eliminazione, equilibrio e fuga. Nella fase di eliminazione, il sistema immunitario individua ed elimina il tumore; nella seconda fase, si instaura un equilibrio dinamico tra i componenti del sistema immunitario e le cellule tumorali, le quali accumulano mutazioni genetiche ma la loro proliferazione risulta limitata. Nell'ultima fase, si verifica progressione tumorale,

grazie alla presenza di cellule tumorali resistenti, selezionate durante la fase precedente. Inoltre, il tumore a questo ultimo stadio è in grado di influenzare negativamente l'azione del sistema immunitario (immunosoppressione tumorale). E' ampiamente riconosciuto che a livello cerebrale l'interazione tra sistema immunitario e tumore è specializzata ed unica. In passato, il cervello era considerato un organo immunologicamente "privilegiato", a causa dell'esistenza della barriera emato-encefalica, della mancanza di convenzionali vasi linfatici e della presenza di diversi fattori immunosoppressivi. Diversi studi hanno dimostrato che cellule del sistema immunitario possono infiltrare la massa tumorale. Il cervello è ora considerato un organo immunologicamente "specializzato".

Immunosoppressione locale associata al glioma. I meccanismi immunosoppressivi associati al glioma possono essere divisi in passivi ed attivi. La cellula tumorale può sottrarsi attivamente al riconoscimento da parte del sistema immunitario sottoesprimendo le molecole MHC I. Essa può indurre inoltre apoptosi di cellule immunitarie esprimendo FasL sulla membrana cellulare. Inoltre, l'ipossia caratterizzante il microambiente tumorale contribuisce a sopprimere le normali funzioni delle cellule del sistema immunitario. Le cellule tumorali possono inoltre secernere fattori solubili immunosoppressivi come IL-10 e TGF-β (immunosoppressione passiva). Le cellule T regolatrici (T_{reg}) rivestono infine un ruolo essenziale nell'immunosoppressione. Esse possono secernere citochine inibitorie e/o modulare la funzionalità delle cellule presentanti l'antigene. Inoltre, possono presentare citotossicità.

Immunosoppressione periferica associata al glioma? In questo lavoro sperimentale, abbiamo analizzato se il glioma può indurre immunosoppressione anche a livello periferico. Per questo scopo, abbiamo utilizzato un modello murino di astrocitoma. Il modello utilizzato è transgenico e sviluppa il tumore in modo spontaneo, riproducendo quello che avviene nell'uomo. E' stato infatti dimostrato che le mutazioni genetiche e l'eterogeneità del modello riproducono la progressione e le caratteristiche del glioma umano. Inoltre, il transgene contiene il gene LacZ, il quale codifica per un antigene conosciuto (β-galattosidasi), considerato un antigene tumore-associato.
Studi su pazienti affetti da glioma hanno dimostrato diverse disfunzioni nella risposta immunitaria a livello periferico; una conseguenza di queste è la dimostrata anergia cutanea a comuni antigeni batterici. Inoltre, è stato osservato che alcuni tipi cellulari del sistema immunitario di pazienti malati sono affetti da anomalie a livello quantitativo e funzionale. In particolare, il rapporto T

linfocitario CD4/CD8 risulta diminuito; inoltre, la responsività dei linfociti T alla stimolazione *in vitro* con mitogeno risulta ridotta. Non sono invece state dimostrate alterazioni nelle proporzioni dei linfociti B. E' però da considerare il fatto che questi studi non hanno valutato la possibile concomitante terapia somministrata ai pazienti analizzati e sono stati effettuati solamente durante gli ultimi, sintomatici stadi della malattia. L'utilizzo di un modello risulta vantaggioso principalmente perché permette lo studio dell'immunologia tumorale anche ai primi stadi della malattia e l'analisi del modello evita l'influenza che il trattamento terapeutico ha sul sistema immunitario.

In questo lavoro sperimentale, abbiamo determinato quantitativamente diverse tipologie di cellule immunitarie in milza e linfonodi inguinali, per determinare se significative alterazioni interessano i topi portatori di tumore. Abbiamo inoltre eseguito un test *in vitro*, per analizzare se la risposta dei linfociti T splenici alla stimolazione con mitogeno è diminuita come osservato nei pazienti. Infine, abbiamo indagato la capacità di attivazione dei linfociti T in topi transgenici, dopo vaccinazione con un virus ricombinante. Abbiamo inoltre analizzato la loro risposta specifica *in vivo* ad un antigene tumore-associato.

Stato immunologico periferico nel modello spontaneo di astrocitoma. Abbiamo osservato che il bilancio immunologico non è alterato a partire dai primi stadi della malattia, ma solo nelle ultime fasi della progressione tumorale. Il rapporto T linfocitario CD4/CD8 è infatti risultato significativamente diminuito solo nel topo malato. Abbiamo inoltre dimostrato che le cellule soppressive di origine mieloide (gruppo eterogeneo di cellule immunosoppressive) non sono presenti in aumentate quantità a livello splenico in topi affetti da glioma, come invece dimostrato in altri modelli. Come osservato nell'uomo, anche nel modello murino di glioma la porzione periferica dei linfociti B non risulta alterata. La risposta dei linfociti T alla stimolazione *in vitro* si è dimostrata mitogeno dose-dipendente. Non abbiamo osservato iporesponsività dei linfociti T provenienti da topi malati, ma è da considerare il fatto che il test eseguito è *in vitro* e di conseguenza non è stato riprodotto lo stesso ambiente immunosoppressivo presente *in vivo.* Recenti studi hanno infatti dimostrato una correlazione tra presenza del glioma e anormalità dei linfociti T. Abbiamo infine eseguito una vaccinazione con un virus ricombinante: VV-LacZ. Abbiamo analizzato in primo luogo la capacità dei linfociti di attivarsi. Nel topo transgenico, la capacità di attivazione delle suddette cellule non risulta diminuita. Non è stata invece osservata una risposta specifica all'antigene tumore-associato nei topi transgenici, dopo vaccinazione e stimolazione *in vitro*. Da discutere è l'efficienza dello

strumento di vaccinazione utilizzato, il quale è stato dimostrato susciti un'elevata risposta virus-specifica ed una modesta risposta immunologica rivolta verso il gene codificato nel costrutto. Infine, la stimolazione *in vitro* è stata effettuata con due differenti peptidi dell'antigene e da esaminare è la loro immunogenicità.

Conclusioni. Abbiamo dimostrato che la presenza del glioma non influenza in modo significativo lo stato immunologico periferico del modello di astrocitoma utilizzato, eccetto durante gli ultimi stadi della malattia (quando la porzione di linfociti T CD4 risulta compromessa). Inoltre, non abbiamo osservato alterazioni nella responsività e nella capacità di attivazione dei linfociti T in topi affetti dal tumore. Nel contesto del glioma umano, coi dati ottenuti si può ipotizzare che l'immunoterapia è efficace solo se somministrata nelle prime fasi del tumore. La risposta immunologica specifica anti-glioma deve evidentemente avvenire a livello locale, e questo aspetto può rappresentare un fattore limitante per l'efficacia clinica della vaccinazione terapeutica testata.

ABBREVIATIONS

ACK: ammonium cloridium lysis buffer
Ag : antigen
APC : antigen-presenting cell
BBB : blood-brain barrier
BTSCs : brain tumour stem cells
CFSE : carboxyfluorescein succinimidyl ester
cLN : cervical lymph node
CNS : central nervous system
ConA : concanavalin A
CTL : cytotoxic T lymphocyte
DC : dentritic cell
EGFR : epidermal growth factor receptor
FasL : Fas Ligand
FCS : fetal calf serum
FWB : flow cytometer wash buffer
FWB fix : flow cytometer wash buffer with fixative
GBM : glioblastoma
GFAP : glial fibrillary acidic protein
GIM : glioma-infiltrated macrophages
HBSS medium : Hanks Balanced Salt Solution
IL : interleukin
Iono : ionomicyn
LN : lymph node
MDSCs : myeloid-derived suppressor cells
MHC : major histocompatibility complex
NK : natural killer
NTG : non transgenic
PCR : polymerase chain reaction
PGE_2 : prostaglandin E_2
PMA : phorbol 12-myristate 13-acetate
RasB8 mice : GFAP-V^{12} HA-*ras* B8 mice
RPMI : Roswell Park Memorial Institute medium
SD : standard deviation

TAAs : tumour-associated antigens
TG : transgenic
TGF-β : transforming growth factor β
Th_2 : T helper type 2
TLR : toll-like receptor
Th_1 : T-helper type 1
TMZ : temozolomide
TNF : tumour necrosis factor
T_{reg} cells: regulatory T cells
VEGF: vascular endothelial growth factor
VEGFR : vascular endothelial growth factor receptor
VV : Vaccinia virus
WHO : World Health Organization
wo : week-old
βgal : β-galactosidase

1. INTRODUCTION

1.1. GLIOMA

1.1.1. Classification, histopathology and origin of glioma

Glioma, a malignant tumour affecting glial cells, presents some of the greatest challenges in the management of cancer patients worldwide. Even with aggressive surgical resections, along with recent advances in radiotherapy and chemotherapy, the prognosis for patients remains very poor (Kanu et al., 2009).

The World Health Organization (WHO) published in 2007 the fourth edition of the Classification of Tumours of the Central Nervous System (CNS), in which the categorization and the different grades of tumours are clearly described (Louis et al., 2007). The classification is relevant in terms of possible therapies and possible outcome. It permits also to have a worldwide definition of the tumour, based on histopathological and clinical diagnostic criteria, in order to permit epidemiological studies and clinical trials without considering national boundaries. There are four different types of glioma: astrocytoma, ependymoma, oligodendroglioma and oligoastrocytoma. In the brain, glial cells are non-neural cells that perform several essential functions, such as anchoring to neurons and regulating the trafficking of molecules and ions through them (astrocytes), covering the cavities of the CNS (ependymal cells) and forming myelin (oligodendrocytes). The WHO classification, depending on the presence of histological features of malignancy, divides tumours in four different prognostic grades. The grade I tumours are surgically curable tumours with low proliferative potential, grade II show low-grade malignancy and are infiltrative, grade III are malignant and require radio and/or chemotherapy.

Glioma usually manifests itself as a focal lesion with central necrosis surrounded by an angiogenic tumour rim; it can be roughly separated into an angiogenic component and an invasive or migratory one. Migration of glioma cells in brain parenchyma relatively far away from the tumour core is common, complicating surgery and radiotherapy (Kanu et al., 2009). In Wen et al. (2008), the histological features of malignant glioma were described. Nuclear pleomorphism, dense cellularity, mitotic figures, pseudopalisading necrosis and vascular endothelial proliferation are observed. Heterogeneity is considered one of the hallmark characteristics of the tumour. Unlike other solid tumours, glioma rarely metastasizes to locations out of the CNS (de Vries, 2009).

Recently, studies about the cell of origin of brain tumours have stimulated new hypotheses. The discovery of the presence of a small subpopulation of brain

tumour stem cells (BTSCs) has led to the hypothesis that neural stem cells are the candidate cells of origin of CNS tumours. In Vescovi et al., BTSCs are defined through different characteristics: they are pluripotent cells with extensive self-renewal ability *in vivo* and *ex vivo*, they have genetic alterations, aberrant differentiation properties, capacity to generate non-tumourigenic as well as tumourigenic cells and have cancer-initiating ability upon orthotopic implantation. Two different mechanisms have been proposed to explain the origin of the tumour: either the de-differentiation of mature astrocytes or the transformation of the endogenous neural stem cell population. The presence of the cell surface marker CD133 on BTSCs has lately been reported and different studies have demonstrated correlation between the CD133 cells and the resistance of tumour to chemo and radio therapies (Vescovi et al., 2006). Although widely used, the validation of CD133 as a unique glioma stem cell marker is debated. Recent data show that $CD133^{+}$ and $CD133^{-}$ cells share similar tumourigenic properties and the isolation of the formers lacks of specificity (Clement et al., 2009). Therefore, additional characterization of the functional role of BTSCs is needed to better understand tumour initiation, progression and resistance to treatment.

1.1.2. Human Glioblastoma Multiforme: an overview

Glioblastoma multiforme (hereafter GBM or glioblastoma) is one of the commonest brain tumours and one of the deadliest ones. It is an invasive, aggressive and neurologically destructive astrocytoma. It is characterised by a very poor median survival, which has only marginally improved over last several decades, despite new technologies and different treatments used. In the most favourable situations and with standard treatments, less than 10% of patients can survive longer than five years (Stupp et al., 2009). GBM is a grade IV astrocytoma: highly malignant, mitotically active, necrosis-prone neoplasm typically associated with fatal outcome (Louis et al., 2007).

Glioblastoma can be separated into two main subtypes, on the basis of genetic differences and of the median age distribution, even if they are largely comparable from the histological point of view and they respond similarly to conventional therapies (Wen et al., 2008; Ohgaki and Kleihues, 2009). Primary glioblastoma typically occurs in patients of mean age of 62 years, whereas Secondary GBM occurs on average in 45 years-old patients (de Vries et al., 2009). Primary tumours are *de novo* tumours, which show a clinical history of a period < 3 months (68% of cases). Secondary GBM develop more slowly from low-grade astrocytoma (WHO grade II) or from anaplastic astrocytoma (WHO grade III) (figure 1). In the first case, the mean time of progression is estimated to be 5.1

years, for the second case 1.9 years. Progression from WHO grade II through WHO grade III to grade IV GBM is also possible (Kanu et al., 2009).

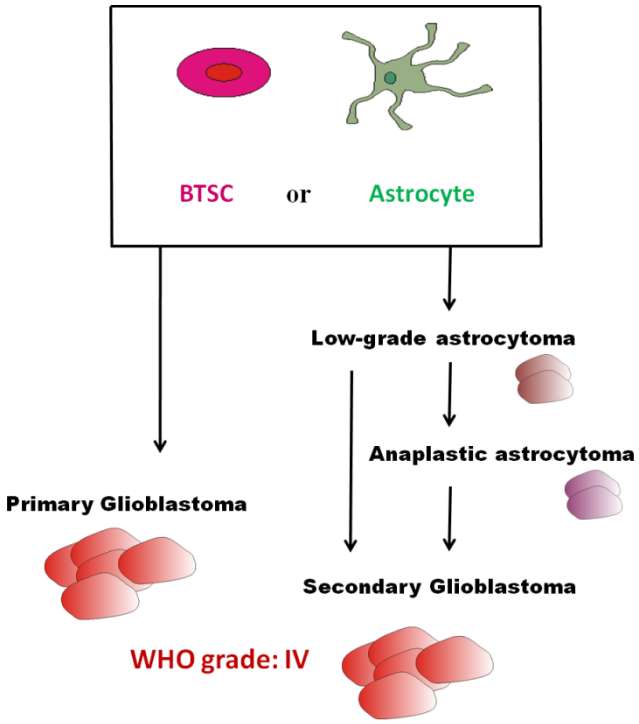


Figure 1: Primary and Secondary Glioblastoma. Glioma is hypothesised to derive from brain tumour stem cells (BTSCs) or from de-differentiated mature astrocytes. In this figure, two different pathways leading to Primary and Secondary Glioblastoma are shown. Primary GBM are *de novo* tumours, whereas Secondary GBM derive from low-grade astrocytoma and/or from anaplastic astrocytoma.

In the United States and in the European countries, the annual incidence of total Primary and Secondary malignant glioma is estimated to be 5 per 10,000 person-years (Wen et al., 2008). The aetiology of the tumour is likely to be multifactorial; genetic and environmental factors can influence the outcome of the tumour. Suspected predisposing factors are exposure to therapeutic ionizing radiation and chemical carcinogens. Only the first one recently has been statistically proven (Kanu et al., 2009). The adverse prognostic factors include advanced age. Other factors are histological features of glioblastoma and unresectable tumour. Furthermore, glioblastoma is more common in men than in women and in whites than in blacks (Wen et al., 2008). The symptoms are different and include peritumoural edema, venous thromboembolism, seizures, fatigue, memory loss, confusion, personality changes, focal neurologic deficits, high intracranial pressure, headaches that when severe may be associated with

nausea and vomiting (Wen et al., 2008; Kanu et al., 2009). Generally, the tumour progression is accompanied by symptoms and preventative screening is not considered useful for asymptomatic patients. The diagnosis is usually performed using magnetic resonance imaging (MRI); when MRI is not possible, computed tomography scan is useful, even if remains inferior in image detail. Furthermore, positron emission tomography, magnetic resonance perfusion and magnetic resonance spectroscopy can be used to delineate levels of metabolites and to discriminate malignant tumour from benign lesion (Wen et al., 2008; Kanu et al., 2009).

It is well-known that neoplasms occur as a consequence of successive accidental mutational events; generally, the alterations concern signalling pathways and cell-cycle regulation. In particular, oncogenes and tumour-suppressor genes play an important role in carcinogenesis; the activation of the former and the deactivation of the latter favour glioma evolution. Many molecular studies have been performed in order to have a better understanding of the genetic mutations in glioma cells. These can help to identify new therapeutic targets and, consequently, to develop new, more efficient therapies. From the molecular profiles point of view, Primary and Secondary glioblastoma are different and are believed to derive from two different genetic pathways (Martinez and Esteller, 2010). Many different genetic mutations characterise the tumour, but only the main ones are considered in this section (table 1).

The epithelial growth factor-receptor (EGFR) becomes activated through the binding of its ligand EGF; afterwards the complex EGFR/RAS/NF1/PTEN/PI3K signalling pathway is initiated, resulting in cell proliferation and increased cell survival. EGFR gene overexpression is generally observed in Primary GBM (approximately 40%), but it is very rare in Secondary tumours (Ohgaki and Kleihues, 2009). The most common mutated variant of EGFR that has been identified is EGFR*v*III; the resulting defective receptor is constitutively activated (Ohgaki and Kleihues, 2009; Kanu et al., 2009; Wen et al., 2009). Even if high levels of RAS-GTP have been documented in primary tumours, specific mutations affecting RAS in GBM have not been detected. RAS overexpression is likely to be caused by upstream factors or by the loss-of-function of the neurofibromatosis 1 (NF1) gene, which encodes for the neurofibromin protein, a RAS negative regulator (Kanu et al., 2009). RAS synergy with other signalling pathways is relevant to give rise to glioma (Munoz et al., 2009). Inactivation of the PTEN gene, a tumour-suppressor gene that regulates the PI3K pathway, is more common in Primary glioblastoma (40%) than in Secondary (Wen et al., 2008). The TP53/MDM2/MDM4/$p14^{ARF}$ pathway is another important pathway involved in the evolution of glioma (Ohgaki and

Kleihues, 2009). Notably, TP53 gene encodes a protein that promotes cell cycle arrest or apoptosis in response to DNA damage, hypoxia or cell cycle abnormalities. It is essential for cellular and genetic stability. TP53 induces transcription of genes such as p21, which prevents the progression through G1 cell cycle phase into S cell cycle phase. Unlike EGFR and PTEN mutations, TP53 alterations are more frequent in Secondary than in Primary glioblastoma. TP53 mutations observed in the two different subtypes of glioblastoma are diverse and this suggests, as previously stated, that the acquisition of genetic alterations may result from dissimilar molecular mechanisms. RB1 controls the transition from G1 into the S phase of the cell cycle by inhibiting the action of elongation factor 2 (E2F), and its expression is in general altered in glioma cells (Kanu et al., 2009). One of the most frequent mutations in GBM is loss of heterozygosity (LOH) on chromosome 10q, very common in both of glioblastoma subtypes (Martinez and Esteller, 2010; Ohgaki and Kleihues, 2009). The IDH1 gene encodes isocitrate dehydrogenase 1, the enzyme that catalyses the oxidative carboxylation of isocitrate to α-ketogluatrate in the Krebs cycle. When mutated, the enzyme loses affinity for its substrate. In low-grade astrocytoma and in Secondary glioblastoma, IDH1 is frequently mutated (> 80%); in contrast, in Primary glioblastoma mutations are rare (< 5%). As Ohgaki and Kleihues (2009) explain, this can be additional evidence of the possible different origins of the two subtypes of glioma. Vascular endothelial growth factor receptor (VEGFR) is over expressed both in Primary and Secondary GBM (Wen et al., 2008).

Genetic and epigenetic mutations	Primary GBM	Secondary GBM
EGFR overexpression	*40%*	*rare*
PTEN inactivation	*40%*	*rare*
TP53 alteration	*rare*	*frequent*
LOH 10q	*5%*	*80%*
VEGFR overexpression	*frequent*	*frequent*
MGMT methylation	*36%*	*75%*

Table 1: Genetic and epigenetic mutations in Primary and Secondary GBM. All different mutations mentioned in this section are listed. Interestingly, alterations observed in the two different kinds of GBM are different. This suggests that different molecular mechanisms lead to different types of GBM, although histology and therapy response is largely comparable between the two.

Epigenetics are also important in glioma evolution (Martinez and Esteller, 2010). An explicative example of a regulator gene undergoing methylation-mediated inactivation in glioblastoma is the DNA repair gene O^6-methylguanine-DNA methyl transferase MGMT), which removes promutagenic alkyl groups from O^6-methylguanine. Reduction or loss of MGMT activity can occur due to the promoter methylation and is associated with improved response to chemotherapy and better prognosis (Ohgaki and Kleihues, 2009). Methylation is more frequently observed in Secondary GBM (75%) than in Primary GBM (36%); in Secondary GBM is often associated with TP53 mutations (Martinez and Esteller, 2010). Martinez and Esteller (2010) have underlined another important epigenetic change that has to been taken into consideration: the expression level of certain micro RNA. Recent studies have shown that miR-21 has a higher expression in GBM in comparison with normal brain; moreover, downregulation of miR-21 in glioma cells can lead to reduction of their invasive capacity.

1.1.3. Glioblastoma prognosis and standard treatment

The current standard of care for GBM patients is maximum surgical resection if feasible, followed by radiotherapy and concomitant systemic temozolomide (TMZ) chemotherapy. TMZ is an oral alkylating agent with antitumor activity and it can cross the blood-brain barrier (BBB) (Kanu et al., 2009). Notably, Stupp et al. (2009) performed a clinical trial on glioblastoma patients, in which radiotherapy alone is compared to radiotherapy and concomitant TMZ chemotherapy followed by adjuvant TMZ chemotherapy (figure 2). The median survival benefit with chemo-radiotherapy is statistically significant and clinically meaningful; chemo-radio is considered preferable to radiotherapy alone. Toxic effects of the currently used therapy include nausea, leucopoenia, anemia, thrombocytopenia, fatigue and severe infections, but all of them are considered acceptable (Stupp et al., 2009). The poorly efficacious outcome of the currently used treatment can be explained by diverse reasons. BTSCs seem to be remarkably resistant to radio and chemo therapies, probably owing to their hallmark genomic profile (Kanu et al., 2009). Furthermore, the standard TMZ chemotherapy fails due to the presence of MGMT (see section 1.1.2.). Recently, combinations of TMZ with MGMT inhibitors and inhibitors of other repair enzymes have studied, in order to improve the efficacy of this therapy (Kanu et al., 2009; Wen et al., 2008).

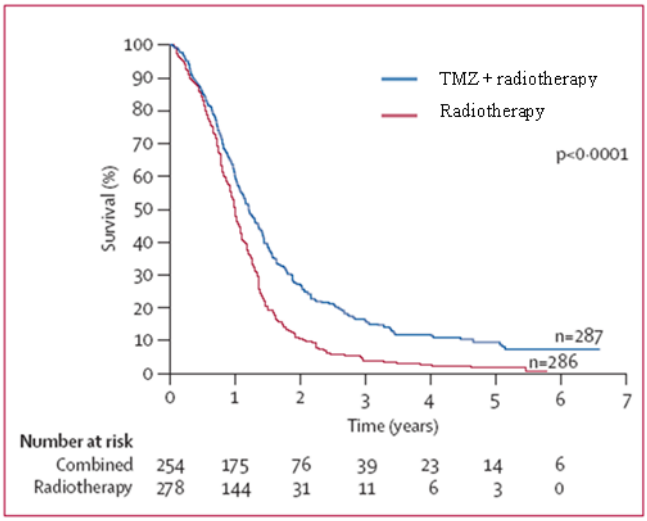


Figure 2: Survival curve of patients treated with combined chemo-radiotherapy. *(Reproduced from Stupp et al., 2009)*. Survival curve in glioblastoma patients treated with radiotherapy alone versus glioblastoma patients treated with radiotherapy with concomitant and adjuvant temozolomide chemotherapy. Survival is greater with combined treatment: a few patients in favourable prognostic categories survive longer than 5 years.

Other different therapies are at present investigated, to improve the prognosis and to ameliorate the quality of life of glioblastoma patients. Bevacizumab (Avastin®), a monoclonal antibody against vascular endothelial growth factor (VEGF), combined with irinotecan, a chemotherapeutic agent, is a new chemotherapy protocol increasingly used for recurrent malignant glioma. To consider is that this combined treatment has many side-effects, such as thromboembolic complications and gastrointestinal toxicities (Xu et al., 2010).

Other types of treatment under investigation are targeted molecular therapy, such as anti-EGFR therapy, immunotherapy (discussed in detailed in section 1.3.6.) and gene therapy; additionally, drugs to overcome resistance are studied. Combinations of different kinds of therapy are required, due to the fact that several pathways can be concurrently altered in glioma cells, which are very heterogenic and may manifest different types of alterations and different capacity of resistance (Kanu et al., 2009).

1.2. CANCER AND IMMUNOLOGY

Most information about the interaction between cancer and immune system has come from tumours in sites other than the brain. In the past, the hypothesis of “immunosurveillance”, which is the detection and elimination of tumour cells by the immune system, has been proposed. Different studies have been performed in immunodeficient mice strains to prove the correlation with the

lack of components of immune system and the develop of the tumour (Swann and Smith, 2007). Nevertheless, evidence of immunosurveillance is rare in clinical settings (Bui and Schreiber, 2007). The concept of "immunosurveillance" has left room for a refined theory, named "immunoediting", which consists of a process divided into three different phases: elimination, equilibrium and escape (Bui and Schreiber, 2007; Swann and Smith, 2007) (figure 3). The elimination corresponds to the mechanism previously described by the "immunosurveillance" theory. After that, a phase of dynamic equilibrium is thought to be established, in which tumour cells can continue to evolve and they probably can accumulate genetic changes. The third phase is characterised by the progression of the tumour, which is now able to resist the action of the immune system, probably thanks to the selection of specific resistance tumour cells variants (Swann and Smith, 2007). There is an ongoing interest to validate or to refute the immunoediting hypothesis, in relevant cancer models and in clinical pathologies.

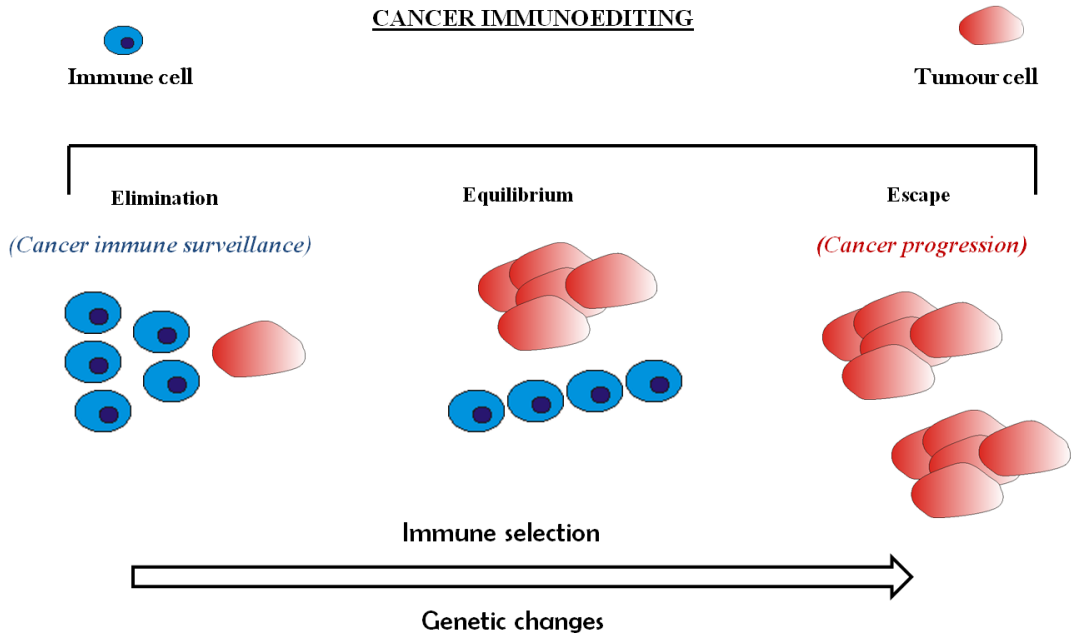


Figure 3: Cancer immunoediting *(adapted from Swann and Smyth, 2007).* Cancer immunoediting is a process divided into three phases. The first one, named elimination, corresponds to cancer immune surveillance. During the equilibrium phase, immune cells and tumour cells are temporary in dynamic equilibrium and tumour cells continue to evolve, accumulating genetic changes. Resistant tumour cell variants are also selected. In the escape phase, immune response fails to completely eliminate the tumour and this results in cancer progression.

Referring in particular to the central nervous system, our understanding of the interaction between immune system and tumour is not so clear; information is frequently extrapolated from other tissues which are indeed different from brain (Walker et al., 2002). It is well-known that immune reactions happening in the brain are specialised and unique (see the 1.3.1.) and further investigations are needed in order to completely understand them.

1.3. IMMUNOLOGY OF THE CENTRAL NERVOUS SYSTEM

1.3.1. The brain: an immunologically specialised site

For many decades, the CNS has been believed to be an immunologically inert site completely separated from the peripheral immune system. It has been defined as an "immunologically privileged" site. Initially, in fact, it was observed that foreign tissue grafts survive for more prolonged periods when placed within the parenchyma of the brain compared with being placed under the skin (Carson et al., 2006). Accordingly, the presence of the BBB, the regulation of the cell trafficking through it, the lack of conventional lymphatics, many local immunosuppressive factors and the absence of resident dendritic cells (DCs) have given support to the hypothesis that the brain is an immunologically inert site (Carson et al., 2006; Grauer et al., 2009). The BBB consists of specialised cerebrovascular endothelium, basement membrane, pericytes and astrocytes. The BBB endothelium has tight junctions, low pinocytotic activity and lacks of transendothelial fenestrations. Primarily, it has been presumed to limit movement of leucocytes and solutes from blood to CNS (Carson et al., 2006). Besides the physical restrictions described, it has to be taken in consideration the fact that the brain is an essential organ for survival and that has to be protected by pathogens; even so, it cannot tolerate volume increase after an inflammatory cellular accumulation, because the skull lacks elasticity (Carson et al., 2006). In addition, inflammatory reactions have to be kept under control in order to avoid their potential capacity to damage neurons (Carson et al., 2006; Walker et al., 2009). Diseases such as multiple sclerosis (MS) and experimental autoimmune encephalitis (EAE), multiple sclerosis model in animals, provide strong evidence that immune cells can act in the brain (Okada et al., 2009). Moreover, the documentation of neuroinflammation in diverse CNS disorders has led to the conclusion that the CNS cannot be considered an immunologically inert, but rather an "immunologically specialised" site.

With respect to brain and glioma, there is much evidence which proves an interaction between immune system and tumour (Walker et al., 2009; Okada et al.,

2009; Grauer et al., 2009). It should be noted that the BBB may be compromised in glioma patients and this can affect immune cells migration: permeability is higher due to increased fenestrations and due to the diminished number of BBB-associated pericytes (Okada et al., 2009). But leukocyte entry into the CNS is generally not absolutely dependent on breakdown of BBB integrity. Tissue-selective trafficking of leucocytes is mediated by unique combination and sequential interactions of adhesion molecules and chemokines (Kunkel and Butcher, 2002). What is unique to the CNS is the specific combination of molecules and receptors used. The translocation of leucocytes occurs by the multi-step model, clearly described in Kunkel and Butcher (2002). This model consists of a first process called rolling in which blood leucocytes interacting transiently and reversibly with the vascular endothelium. The second step is the activation of leucocytes which, after their biding to activation factors on the endothelium surface, express integrins. During the following process named adhesion, leucocytes reversibly arrest and adhere on vascular endothelium. The final step is the transmigration across the endothelium, directed by chemokine gradients.

1.3.2. Brain tumour innate immunity

The immune system is based on two distinct types of responses: innate immunity and adaptive immunity. Innate immunity provides the first defence of our organism against pathogens; it involves recognition of pathogens by receptors encoded by conventional genes, followed by activation of destructive effector mechanisms. The cells that orchestrate the innate immune response are monocytes, macrophages, granulocytes (eosinophils, basophils, neutrophils and mast cells), natural killer (NK) cells and DCs. Also serum proteins of the complement system participate in immune innate defence (Parham, The immune system, 2009).

Adaptive immunity forms the second line of defence and is constituted by B and T cells. It acts in a highly specific way but the receptors are not necessarily pathogen-specific. They are characterised by infinite diversity, because encoded by genes which undergo somatic recombination. During an infection, a small subset of lymphocytes specific for the pathogen is selected and expanded. Adaptive immunity can also provide long-term immunological memory (Parham, The immune system, 2009). The interaction between immune cells of the innate immune system and immune cells of the adaptive immune system is determinant for mounting an effective immune response. As seen in section 1.3.1., the distinctive conditions imposed by the CNS influence the anti-tumour immune response happening in the brain.

Microglia, glial cells that are resident macrophages in the CNS, are considered the first cells which respond to a variety of stress signals in the brain (Okada et al., 2009). In common with the other cells of the innate immune system, they can detect pathogen-associated molecular patterns (PAMPs) by means of pathogen-recognition receptors (PPRs) that include the toll-like receptors (TLRs) (Walker et al., 2009). In glioma, resident microglia are difficult to be clearly distinguished from glioma-infiltrated macrophages (GIMs) apart from by flow cytometry. The GIMs express raised levels of TLRs. GIMs are the largest subpopulation infiltrating glioma, however, differently to microglia, they do not appear to be stimulated to produce pro-inflammatory cytokines (Okada et al., 2009), such as nitric oxide (NO) and tumour necrosis factor (TNF)-α (Prins and Liau, 2004). Both microglia and GIMs are phagocytic cells of myeloid origin with capacity of presenting antigen, but their ability of doing this is much lower than DCs (Grauer et al., 2009). NK cells can mediate cytolysis of infected or transformed cells and secrete INF-γ. In order to induce cytolysis, they require the presence of an activating signal, for example given by the expression of stress-induced proteins by tumour cells, and simultaneously, the absence of an inhibitory signal (reviewed in Walker et al., 2009). In fact, although all nucleated cells normally express major histocompatibility complex (MHC) class I, transformed or virus-infected cells often down-regulate the classical MHC class I expression in an effort to avoid recognition by $CD8^+$ cytotoxic T lymphocytes (CTLs). NK cells can recognise and kill cells which do not present MHC class I (reviewed in Walker et al., 2009). Actually, little is known about the potential impact of NK cells on human glioma (Walker et al., 2009). Also the role of granulocytes in the contest of glioma is not well-characterised in the literature.

Another important cellular component of innate immunity is the dendritic cell. DCs are professional antigen-presenting cells (APC) and can activate naïve T cells and induce their differentiation into T-effector cells. Hence, their role in inducing adaptive immunity is very important. They are present in virtually all tissues and organs and they continuously monitor their environment. Moreover, they can be recruited to the tumour site from the periphery (Grauer et al., 2009). Two different subtypes of DCs exist: plasmacytoid and conventional DCs. The conventional DCs can further be divided in migratory and lymphoid-tissue-resident. DCs, after the capture of the pathogen or antigen and activation via PPRs, undergo a process named maturation and present the processed antigen (Ag) to the T cells in secondary lymphoid organs, using MHC class II and/or MHC class I. Co-stimulatory molecules, such as members of the B7 family, highly expressed by DCs, are needed to obtain full activation of naïve T cells (Walker et al., 2009). Focusing on glioma, the process of presenting tumoural

antigen has been formerly hypothesized to happen through different possible mechanisms: (1) after the uptaken of the Ag, DCs migrate to the cervical lymph node (cLN) and therein presents the Ag to the T cells; (2) Ag itself drains to cLN where it is taken up by DCs and processed; (3) tumoural cells that express Ag may directly drain to cLN and present their own Ag. The first hypothesis is currently supported by several lines of evidence (Okada et al., 2009).

1.3.3. Brain tumour adaptive immunity

The cells that orchestrate adaptive immune responses are T cells and B cells. Both of them are bone marrow-derived cells. They can recognise their cognate antigen through their specific receptor: T cell receptor (TCR) and B cell receptor (BCR). In particular, the portion of the antigen recognised by the T cell receptors is called the epitope, which derives from proteolytic cleavage (Parham, The immune system, 2009).

Concerning B cells, when cognate antigen binds to their receptor, they proliferate and differentiate into plasma cells, which secrete high level of antibodies specific to the precise antigen encountered. Five different classes of antibodies exist: IgG, IgA, IgM, IgD and IgE. They are involved in several immune reactions, such as neutralisation and opsonisation, and they constitute the humoral arm of immunity (Parham, The immune system, 2009). In this section, the attention is focused on T cells rather than on B cells, because the B lymphocytes are probably not a major component of spontaneous immunity to brain tumours (Walker et al., 2009). In fact, B cells poorly infiltrate brain tumours and in addition, the capacity of antibodies to enter the brain is limited.

Immature T cells are selected in the thymus to recognise self-MHC molecules (positive selection) and for the absence of autoreactivity against self-antigens (negative selection). This process makes the immune system tolerant of self. In the context of self tolerance, a key role is played by the transcriptional factor called autoimmune regulator (AIRE), which facilitates expression of different tissue-specific genes by a subpopulation of epithelial cells in the medulla of the thymus. The phenomenon of tolerance is not limited to central (i.e. thymic) tolerance; autoreactive cells which escape deletion by central tolerance can be induced to become anergic or can be suppressed by regulatory T cells in the periphery. Autoimmune diseases are generally caused by defects in self tolerance which result in autoreactive inflammation (Parham, The immune system, 2009).

Immature T cells in the thymus express both CD4 and CD8 co-receptors. After maturation, they become single-positive $CD4^+$ or $CD8^+$ T cells. $CD8^+$ T cells generally recognise epitopes of endogenous origin, presented on MHC class

I. $CD4^+$ T cells recognise epitopes derive from exogenous sources, presented on MHC class II.

$CD8^+$ T cells recognise cognate peptide and the specific target cell if it expresses appropriate MHC class I. They can adopt different cytotoxic mechanisms to induce apoptosis in their target cells. Cytotoxicity can derive from the contents of cytotoxic granules, released by exocytosis in the immunological synapse formed between the CTL and the target cell. The granule contents include perforin, granulysin and granzymes (Parham, The immune system, 2009). The hypothesis that perforin is inserted into the target-cell membrane and creates pores through which granzymes can penetrate the cell has recently been debated in the literature. However, perforin is necessary to mediate the release of granzymes in the cell. Granzymes trigger apoptosis of target cells via the activation of the caspase proteolityc cascade or via caspase-indipendent mechanisms. CTLs can also secrete TNF and lymphotoxins (LTs); they can also induce apoptosis of the target cell through Fas Ligand (FasL) binding (Parham, The immune system, 2009). After antigen presentation in the cLN, CD8 T cells undergo clonal expansion. Using orthotopically implanted glioma mice models, it was demonstrated that CD8 T cell proliferation happens in superficial and deep cLNs and in lumbar lymph nodes (LNs), and it is associated with a rapid up-regulation of the integrin heterodimer $\alpha_4\beta_1$, very relevant for the brain tropism of the activated T cells (Calzascia et al., 2005). Moreover, this study demonstrated that the imprinting of T cells depends on the site in which the antigen is captured, rather than the particular LN in which T cell proliferation occurs (Calzascia et al., 2005). When CD8 T cells reach the tumour site, they further differentiate in the brain, exhibiting enhanced INF-γ and Granzyme B expression and induction of $\alpha_E\beta_7$ integrin expression that facilitates T cell retention in the brain (Masson et al., 2007). INF-γ, a type II interferon, activates macrophages and increases the processing and presentation of antigens (Pharam, The immune system).

Naïve $CD4^+$ T-cells can differentiate, depending upon the stimulus, into T-helper type 1 (Th_1), T-helper type 2 (Th_2), Th_{17} cells or regulatory T cells (T_{reg} cells). CD4 T cells are called "helper cells" when they secrete a variety of cytokines that enhance the function of other cells (Prins and Liau, 2004). Generally, $CD4^+$ T cells can help B cells make antibodies, they induce macrophages to develop enhanced microbicidal activity, and they recruit neutrophils, eosinophils and basophils to sites of infection (Parham, The immune system, 2009). Defining the role of $CD4^+$ T cells in brain tumour immunity is complicated, due to the fact that they can either manifest pro- or anti- tumour effects. Besides, very few glioma antigens recognised by $CD4^+$ T cells have been identified (Walker et al., 2009).

1.3.4. Tumour immune escape

Despite the observation that immune cells can infiltrate the tumour in glioma patients (see section 1.3.1.), the role of the immune system in tumour progression is still debated. In section 1.2., the theory of "immunoediting" is explained; according to this concept, the tumour can overwhelm immune response due to selection of resistant tumour cell variants. The escape of the tumour from the immune system response is usually defined as tumour immune escape. Tumour escape permits the progression of the tumour to advanced stages (Cao, 2009), thus understanding the mechanism is a major challenge for cancer research.

Glioma immune escape mechanisms can be divided into two different groups: passive and active. The passive mechanisms are represented by intrinsic properties of glioma cells. Indeed, glioma may attempt to passively escape immune detection by downregulation of MHC expression; furthermore, areas of hypoxia represent a hostile microenvironment for immune cells (Walker et al., 2009). The immunosuppression actively elicited by glioma concerns soluble factors, cell surface molecules and immunosuppressive cells (Walker et al., 2009; Grauer et al., 2009; Okada et al., 2009). As a consequence, in the tumour microenvironment the normal function of immune effector cells is profoundly suppressed (Grauer et al., 2009; Tran Thang et al., 2010) and the presence of immunostimulatory molecules such as interleukin (IL)-12 and INF-γ is lacking (Grauer et al., 2009; Tran Thang et al., 2010). The glioma microenvironment is composed of tumour cells, endothelial cells, intermingling glia, neurons, extracellular matrix fibers, soluble mediators and a variety of leukocyte subsets (Okada et al., 2009).

One of the most extensively studied glioma-derived immunosuppressive factors is Transforming Growth Factor (TGF)-β2, originally called glioblastoma cell-derived T-cell suppressor factor (Walker et al., 2002). TGF-β2 is an isoform of TGF-β in mammals; the isoforms TGF-β1, 3 and 4 are differentially expressed. TGF-β2 has multiple and complex effects; in particular it inhibits maturation and antigen presentation by DCs or other APC (Okada et al., 2009) and also suppresses NK and T-cell proliferation (Walker et al., 2009). It has been demonstrated that TGF-β2 is produced by glioma cells and by glioblastoma *in vivo* (reviewed in Walker et al., 2009). All these characteristics make TGF-β2 an attractive target for novel therapeutic approaches and the most advanced in the clinical application is the use of an antisense oligonucleotide (Grauer et al., 2009). Other immunosuppressive soluble glioma origin-factors are gangliosides, prostaglandin E_2 and IL-10 (Walker et al., 2002). Gangliosides (GANGs) are sialic acid-containing glycosphingolipids present in human plasma (Dix et al.,

1999). GANGs can modulate lymphocytes responsiveness; it can be speculated that immunosuppression by GANGs might account for some of the dysfunctions observed in glioma patients (Dix et al., 1999). Prostaglandin E_2 (PGE_2) is a product of arachadonic acid metabolism and it is produced at the sites of inflammation or tissue damage where it can enhance the vascular permeability (Dix et al., 1999). The role of PGE_2 in glioma tolerance has not been clarified yet and further characterizations are needed. In fact, PGE_2 can be associated with the suppression of T-cell function and is synthesized by glioma (Dix et al., 1999). However, the concentration of PGE_2 observed in glioma-cell supernatant is not comparable to the concentration required to inhibit T-cell proliferation (reviewed in Okada et al., 2009). Glioma cells synthesize and secrete IL-10; mRNA levels of IL-10 are higher in high-grade astrocytoma rather than in low-grade astrocytoma (reviewed in Dix et al., 1999). IL-10 also has been shown to reduce the antigen presentation capacity of monocytes by down regulating MHC class II expression (Dix et al., 1999).

With respect to the cell surface molecules expressed on glioma cells, FasL has been proposed to contribute to tumour immune escape. FasL belongs to the Tumour Necrosis Factor (TNF) family and it is expressed on glioma cells *in vivo* and *in vitro* (Walker et al., 2009). Its receptor is Fas. The interaction of the latter, expressed on Fas-positive cells, with FasL, leads to a complex cascade of intracellular events ending with cell apoptosis (Okada et al., 2009). The expression of FasL on glioma cells can induce apoptosis of Fas-positive T-cells that infiltrate into the tumour tissue (Okada et al., 2009). The microenvironment can influence the consequences of FasL expression by tumour cells: it has been hypothesized that tumours expressing both FasL and TGF-β2 may be particularly advantaged to overwhelm the action of CD8 T-cells (Walker et al., 2002). Other two cell surface molecules proposed to facilitate glioma immune escape are CD70, a member of the TNF family, and Human Leukocyte antigen (HLA)-G, a non classical MHC class I molecule (Okada et al., 2009).

Immunosuppressive cells play an important physiological role in regulating autoimmunity and controlling inflammation. In some cases, they can be recruited or activated by factors produced by tumour cells, promoting tumour escape (Walker et al., 2009). T_{reg} cells represent a subpopulation of CD4 T-cells and are the most studied immunosuppressive cells in cancer research (Cao, 2009). A large numbers of T_{reg} cells have been found either in circulation or in the tumour micro-environment of patients with various cancers, such as lung, breast, gastric, colorectal cancer and melanoma (reviewed in Cao, 2009). Also in patients with malignant glioma, the T_{reg} fraction is greatly increased (Fecci et al., 2006). Two populations of T_{reg} exist: natural T_{reg} ($n$$T_{reg}$) are produced in the thymus,

whereas induced T_{reg} (iT_{reg}) derive from CD4 naïve T cells which differentiate under the influence of certain cytokines, in particular IL-2 and TGF-β (Walker et al., 2009). nT_{reg} normally migrate to the periphery and account for 10-15% of $CD4^+$ lymphocytes (Cao, 2009). Distinguishing the functions of these two populations and their relative contribution to the comprehensive action of T_{reg} in humans and in experimental animals is challenging (Cao, 2009). $FoxP3^+$ is a useful T_{reg} marker. It is a transcription factor and therefore it is inaccessible to antibodies *in vivo*, largely used for depletion in experimental studies with mouse models (Walker et al., 2009). The function of T_{reg} in immune homeostasis is exemplified by the immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) human disease. IPEX is caused by mutations in the gene FOXP3; as a result, the product of this gene is non-functional and this results in autoimmunity and inflammation. The mouse genetic equivalent for this disease is the so-called *Scurfy* mouse. The impact of $FOXP3^+$ mutation either in humans or in mice underlines the critical role of the transcriptional factor Foxp3 in controlling autoimmune and immunopathologic responses (Walker et al., 2009). It is likely that T_{reg} cells utilise multiple mechanisms to suppress protective tumour immunity. The first mechanism considered is the secretion of inhibitory cytokines, in particular IL-10, TGF-β and IL-35 (Cao et al., 2009). Diverse studies have been performed to better understand the role of these cytokines, either *in vivo* or *in vitro*. Previously in this section, the role of IL-10 and TGF-β, also produced by glioma cells, has been explained. IL-35 has been recently investigated in immune suppression. The role of this cytokine in T_{reg} function in the tumour setting has not been reported (Cao, 2009) and human studies demonstrated that T_{reg} cells do not express IL-35 constitutively (reviewed in Cao, 2009). A second mechanism of action of T_{reg} cells is the use of perforin/granzyme-mediated cytotoxicity. This pathway is known to be utilized by $CD8^+$ T cells and NK cells to kill host cells infected by pathogens or transformed tumour cells. In addition to these two mechanisms, T_{reg} cells may also suppress immune responses by modulating APC. In a transgenic EAE model, it has been reported that the presence of T_{reg} attenuates the establishment of stable contacts between effector T cells and DCs during T cell priming in the lymph nodes (reviewed in Cao 2009). Another type of immunosuppressive cell which is considered to have a role in glioma escape is the myeloid derived suppressor cell (MDSC). Also mesenchymal stem cells, a phenotypically heterogeneous cell population, are immune suppressive cells. Normally, they have anti-inflammatory or anti-proliferative effects on immune cells, but they increase glioma proliferation (Walker et al., 2009).

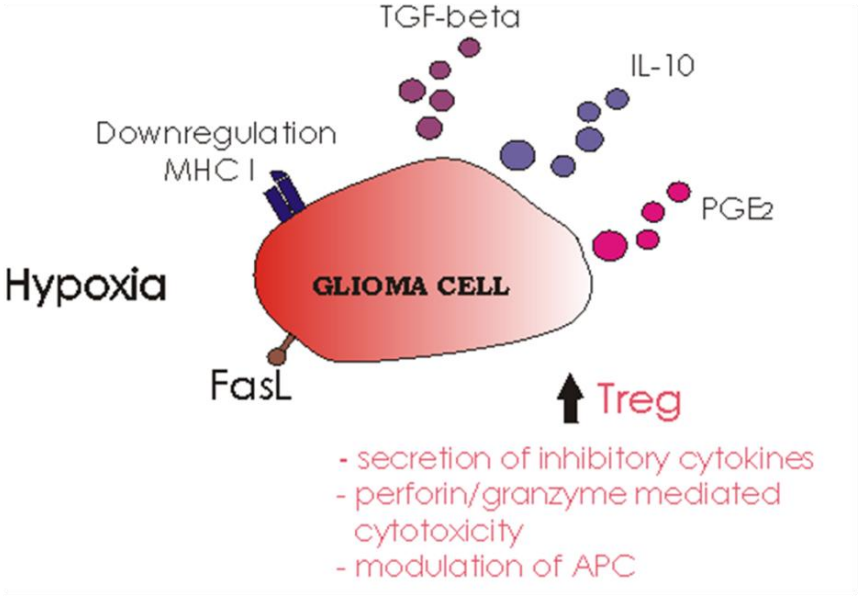


Figure 4: Different mechanisms of glioma immune escape. Passive and active tumour immune escape mechanisms are shown in this figure. Glioma cell down regulates MHC class I molecules expression. Furthermore, FasL is present on cell surface. Hypoxia in tumour microenvironment contributes to suppress normal functions of immune cells. Glioma cell actively secretes soluble immunosuppressive factors (TGF-β, IL-10 and PGE_2 are represented). Immunosuppressive Treg cells also play an important role in glioma immune escape. Their functions are briefly illustrated in the figure.

1.3.5. Impaired immune function in glioma patients

Immunosuppression is frequently associated with malignancy. In glioma patients, although the tumour remains in the intracranial compartment, immunosuppression is both systemic and profound (Fecci et al., 2006). As previously discussed, glioma can adopt different mechanisms to escape immune system responses and to influence the normal activity of immune cells. Proliferative hyporesponsiveness of peripheral blood T cells in response to mitogen stimulation *in vitro* was shown in glioma patients (reviewed in Waziri, 2010). The decrease in responsiveness is variable; tumour size and not location influences the observed amount of immunosuppression (Morford et al., 1997). Additionally, reduced secretion of IL-2 and the presence of defective IL-2 receptor (IL-2R) on T cells characterised glioma patients (Dix et al., 1999). Specifically, proliferative dysfunction is manifested within the $CD4^+$ T-cell subset (reviewed in Fecci et al., 2006). T cell abnormalities are temporarily reversed with tumour resection and return with tumour recurrence, potentially confirming an association between tumour and the suppressive phenotype (reviewed in Waziri, 2010). Severe T cell lymphopenia is another frequently observed defect in patients

bearing glioma (reviewed in Dix et al., 1999). Lymphopenia involves T cells, whereas B cells percentage and absolute number in the peripheral blood of patients are normal (reviewed in Dix et al., 1999). With respect to the percentages of T cells, the number of $CD4^+$ T cells obtained from patients is reduced in comparison to the number of $CD8^+$ T cells. The normal CD4/CD8 T cells ratio is consequently shifted, resulting closer to 1, rather than the usual 2:1 (reviewed in Waziri, 2010). T_{reg} frequently represents an important fraction in the CD4 compartment (Fecci et al., 2006). Furthermore, cytokine production is aberrant. Th_2 cytokine production in glioma patients is usually at higher levels than the production of Th_1 cytokines (Fecci et al., 2006). Th_1 CD4 T cells mediate immune responses against intracellular pathogens, and they produce INF-γ, IL-2 and lymphotoxin α (LTα). Th_2 cells mediate immune responses against parasites and are involved in allergy and asthma; they secrete IL-4, IL-5, IL-13, IL-25 and IL-10 (Parham, The immune system, 2009). Another hallmark of glioma patients is diminished delayed type hypersensitivity (Walker et al., 2002). Moreover, in glioma patients T_{reg} negatively regulates cellular immunity. In peripheral blood, T_{reg} fraction is increased and it correlates with T cell proliferative defects.

Although the mass of information on immune function in patients strongly supports the idea of immune impairment, there are certain issues that remain. In general, the studies on glioma patients peripheral immune status did not report and did not take into account the treatment they were receiving, which interferes with immune cell functionality and viability. Furthermore, all data refer to later stages of the disease, corresponding to the symptomatic phase when patients are studied, and give no information on the origin of glioma systemic immunosuppression. Many tests are *in vitro*, and thus do not necessary recapitulate *in vivo* immune function. Additionally, the studies are limited to blood samples, because tissues such as spleen or lymphoid tissues are not readily available in patients.

1.3.6. Glioma antigens and immunotherapy

Immunotherapy aims to induce antitumour immune response leading to the eradication of malignant cells (Grauer et al., 2009). The understanding of the particular circumstances required and the identification of the immune cells that are able to perform it are essential in developing efficient strategies for cancer therapy (Walker et al., 2009).

In order to obtain efficient and safe immunotherapy, immune cells have to identify correctly and specifically tumour-associated antigens (TAAs) (Prins et al., 2004). Evidently, an ideal target for immunotherapy is an antigen that is

specifically and stably expressed by the tumour and absent in normal tissues (Grauer et al., 2009). Importantly, TAAs used in immunotherapy have to be immunogens, namely they induce immune responses. Advances in molecular genetics and immunology have enabled the identification of several human glioma antigens (Prins et al., 2004). One of the most intensely studied tumour-specific antigens expressed by human glioma is EGFR*v*III (Kanu et al., 2009), expression of which is derived from the oncogenic transformation process. Three principal diverse approaches of immunotherapy can be investigated: passive, active and adoptive (Grauer et al., 2009).

Passive immunotherapy consists of using a variety of effective molecules, including monoclonal antibodies (Okada et al., 2009). Specific monoclonal antibodies can be conjugated with radionucleotides and recognise TAAs (Okada et al., 2009). Notably, with respect to brain tumour, antibodies have the disadvantage to be quite limited in penetrating into the tumour tissue (Grauer et al., 2009).

Active immunotherapy, which can also be defined as tumour vaccination, is an active immunization of patients with the aim to induce potent antitumour responses *in vivo*. In order to induce specific T cell responses, peptide vaccines or recombinant bacterial or viral vaccines can be used (Walker et al., 2009). To obtain reproducible, effective, long lasting anti-tumour response, the utilisation of adjuvants is required. Adjuvants enhance the immune response by prolonging the time of exposure to antigen and by increasing the activity of APCs (Walker et al., 2009).

Adoptive immunotherapy consists of adoptive transfer of effector T cells harvested from patients, activated and expanded *in vitro*. Using this technique, optimal conditions for the culture and amplification of these cells is provided, in absence of tumour-derived immunosuppressive factors (Grauer et al., 2009).

1.4. MOUSE MODELS TO STUDY BRAIN TUMOURS

1.4.1. Animal models in tumour immunology

Brain tumour models, reflecting different characteristics of human glioma, are relevant for the evaluation of new potential therapies. They also represent an essential tool for understanding complex molecular pathways occurring in brain tumours. The features of a robust glioma model include (1) tumours arise from glial cells, (2) similar molecular pathogenesis and similar molecular profiles to human glioma, (3) responsiveness to known therapeutics, (4) predictable development of tumours at a high incidence (Munoz et al., 2009). Importantly,

focusing on glioma progression and its interactions with the immune system, the use of models allows the study of the tumour even at early stages. In contrast, patients cannot be studied until the clinical diagnosis, which is usually made during the later stages of the disease. Furthermore, there are several problems and limitations in studying the immunology of glioma in patients, because they are usually undergoing a specific treatment which interferes with immune responses. Also tissues such as human brain or lymphoid tissue are not readily available.

1.4.2. Murine models of glioma

Different kinds of murine models can be utilised in brain tumour immunology; in particular, grafted models and genetically engineered mouse models (GEM) are described in this section. Human glioma xenografted into immunodeficient mice, although commonly used, will not be considered here because they exclude the study of physiological immune interactions. In contrast, in fully syngeneic grafted mouse models and in GEMs, interactions between tumour and immune system can be studied. The most appropriate model to use has to be chosen according to the questions the specific research addresses.

In grafted models, cell lines from individuals of the same strain are implanted by intra-cerebral injection. This kind of model has predictable features and kinetics, but it does not mimic tumour heterogeneity, the hallmark feature in glioma. In grafted models, tumour is usually homogeneous and has not accumulated multiple genetic defects as happens in humans. Furthermore, the implantation of tumour is an artificial process and influences several *in vivo* parameters. In fact, the injection itself can affect the integrity of the BBB and immune responses may not occur as gradually as in the spontaneous pathology.

Genetically engineered glioma mouse models have been created introducing some of the same genetic abnormalities seen in humans. They can be generated by introducing germ-line or somatic modifications (de Vries et al., 2009). Germ-line models, in particular, give the possibility to obtain spontaneous tumours. They are based on loss or increased expression of relevant genes implicated in human glioma pathogenesis and they also provide a unique opportunity to examine pathological alterations associated with tumour development (Shannon et al., 2005). In these models the integrity of brain and of the BBB is only disrupted subsequent to tumorigenesis.

1.4.3. RasB8 glioma model

GFAP-V^{12} HA-*ras* B8 (referred to hereafter as RasB8) is a germ-line genetically engineered astrocytoma mouse model. In this thesis, we utilised two different genetic backgrounds of the strain: RasB8-CD1 and RasB8-129 (see section 2.1.1.). RasB8 model is characterised by the presence of the transgene V^{12} HA-*ras*:IRES-LacZ, the expression of which is regulated by the astrocytes-specific glial fibrillary acid protein (GFAP) promoter (Ding et al., 2001).

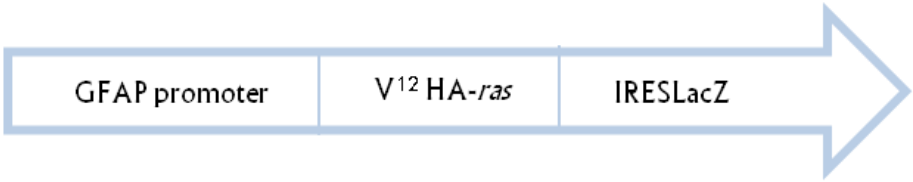


Figure 5: The transgenic construct in RasB8 mice *(adapted from Ding et al., 2001).* The specific GFAP promoter drives the expression of ^{12}V HA-*ras* gene. IRES-LacZ (which encodes for β-galactoside protein) is also under the control of the same promoter.

The technique to obtain RasB8 mice used the transfection of a retrovirus, which carries the gene of interest and a selection marker, into cultured mouse embryonic stem (ES) cells. After positive selection, cells undergo aggregation and are transferred to a pseudo-pregnant female mouse to create chimeric embryos. The chimeric mice are then bred to normal mice and mice which incorporated the transgene in their germ-line generate transgenic offspring (Munoz et al., 2009). The overexpression of V^{12} HA-*ras* gene, although not a primary molecular aberration of human astrocytoma, can initiate the sequence of genetic events that lead to the development of astrocytoma in mice (Munoz et al., 2009). It was also demonstrated that astrocytoma formation is dependent on V^{12} HA-*ras* gene dosage (Ding et al., 2001). There is evidence that RasB8 mice exhibit similarities in pathological and molecular progression features to human astrocytoma (Shannon et al., 2005; Ding et al., 2001). In fact, the mouse astrocytoma is composed of GFAP-positive, highly mitotic, pleomorphic and infiltrative astrocytes, also associated with increased vascularity and necrosis (Ding et al., 2001). Besides, several genetic alterations associated with human low- and high- grade astrocytoma are noted in this mouse model. They present gain-of-function mutations as evidenced by increased expression of EGFR and VEGFR, as well as

loss-of-function alterations, including changes in Tp53 expression or TP53 mutations (Shannon et al., 2005).

The development and the progression of astrocytoma in RasB8 model are heterogeneous in frequency, latency, growth and location; this heterogeneity is reminiscent of human tumour heterogeneity. RasB8 tumour progression was examined by Shannon and colleagues (2005). At birth RasB8 transgenic mice appear normal and astrocytes are not transformed. Glial hyperplasia is the first distinguishing feature in 3-week-old mice. From 3 to 8 weeks, the incidence of low-grade astrocytoma progressively increased. 80% of 12-week-old mice harbour low- or high- grade astrocytoma, 50% of mice die by 4 months.

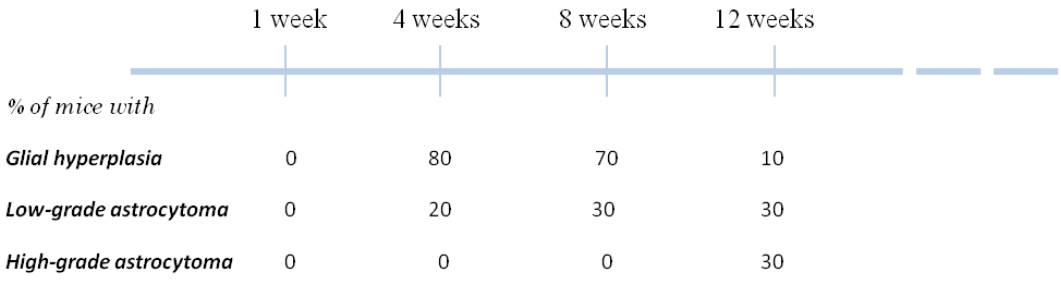


Figure 6: Astrocytoma progression in RasB8 mice *(adapted from Shannon et al., 2005).* The development of the pathology is analysed at different time points. Glial hyperplasia is observed in the majority of the 4 week-old mice; low-grade astrocytoma progression is gradual. High-grade astrocytoma is found in 30% of the 12 week-old mice.

1.5. AIM OF THE PROJECT

Glioma is one of the most aggressive and deadliest brain tumours. Different new therapeutic approaches have led to an improvement in survival, but prognosis still remains poor. This study focuses on glioma immunology. Different studies demonstrated immunosuppression in patients, both at the local and at the peripheral level. However, as discussed, whether this occurs at early stages of disease, and whether it is independent of treatment status is unclear. In this project, we used a spontaneous astrocytoma mouse model, RasB8, in order to investigate peripheral immune status.

Three principal issues were addressed in this thesis:

1) Do RasB8 mice display an altered peripheral immune phenotype and is this reflected in the CD4/CD8 T cell ratio?

2) Do RasB8 T cells show impaired functional responses once removed from the potentially immunosuppressive *in vivo* environment (i.e. *in vitro*)?

3) Are RasB8 T cells able to exhibit functional responses *in vivo*, after vaccination?

2. MATERIALS AND METHODS

2.1. MICE

2.1.1. Strains and breeding

Hemizygous transgenic GFAP-V^{12} HA-*ras* B8 (RasB8) mice (kindly supplied by Dr A. Guha), expressing a single allele of oncogenic V^{12} HA-*ras*, were used. The strain was previously described (section 1.4.3). Other two mouse model strains used in this study are 129 S2/SvPasCr1 (MHC haplotype $H2^b$) inbred strain (Charles Rivers) and C57BL/6 (MHC haplotype $H2^b$) inbred strain (Charles Rivers). Also outbred mouse strain CD1 (Charles Rivers) was bred at our institution. RasB8 mice were maintained on the CD1 background (hereafter RasB8-CD1); S2/SvPasCr1 mice were back-crossed with RasB8 mice and mice from seventh to tenth generation were utilised in this study. Both RasB8-CD1 and 129 S2/SvPasCr1xRasB8 (hereafter RasB8-129) mice were screened for the presence of the oncogene V^{12} HA-*ras*. Mice were sacrificed by isoflurane inhalation. All procedures adopted were approved by the Institutional Ethical Committee and the Cantonal Veterinary Office (Suisse).

RasB8-CD1 and RasB8-129 mice were followed weekly for the development of symptoms. They were considered ill when exhibit more than one of the following symptoms: loss of weight, ruffled fur, debility, irregular breathing, convulsions, difficulty of movement, hunched posture.

2.1.2. Polymerase Chain Reaction: general principles

The Polymerase Chain Reaction (PCR) is a molecular biology technique which permits the amplification of a precise fragment of DNA called "target DNA" starting from DNA termed "template DNA". The enzyme which catalyses this reaction is a thermostable DNA polymerase, isolated from the $Gram^-$ bacteria *Thermus aquaticus*. Oligonucleotides complementary to the sequences which flank the target DNA are required as primers to specifically direct the polymerase to copy the sequence of interest. Primers provide a free 3'-hydroxyl group. In the reaction mix, deoxyribonucleotides (dNTPs) are also needed. PCR is based on thermal cycling and is divided in different stages: denaturation, annealing and extension. Each step is repeated from 30 to 40 times. In the first stage, the template DNA is denaturated by heating above 90°C. The two strands of DNA separate and the target DNA is made accessible. During the annealing stage the temperature is cooled to around 60°C. During this step, oligonucleotide primers

hybridise with their complementary sites. The DNA synthesis step is termed extension. During this stage, polymerase extends the target sequences. The temperature required for the extension corresponds to the optimum temperature of the enzyme used (commonly around 72°C). After one cycle of PCR, new strands are synthesised and they become templates during the next cycle of the reaction. In this way, PCR provides an exponential increase in the amount of DNA produced. Furthermore, all new strands have a precise length, because polymerase amplifies the target DNA and the regions flanking it. After the reaction, the amplified DNA fragments can be visualised on an agarose gel. The specificity of this technique depends on the design of the two oligonucleotide primers. The sensitivity of PCR is very high, foreign DNA contamination has to be avoided using dedicated equipment.

2.1.3. Genotyping

DNA extraction was performed on crude lysate of mice tails. After 5% isoflurane (Abbott, Baar, Switzerland) inhalation anaesthesia, 0.5 cm of tail was cut. DNA digestion was performed with 200 µl tail lysis reagent (PeqLab Direct PCR) containing 0.3 mg/ml proteinase K (Roche). A 55°C overnight incubation, a 45 minutes 85°C final incubation were performed. Lysates were directly used for PCR reaction or stored at -20°C. PCR amplification used a forward primer located in the GFAP promoter (5'-ACTCCTTCATAAAGCCCTCG-3') and a reverse primer located in the 3' region of the HA-*ras* cDNA (5'-CTCGAATTCTCAGGAGAGCACACACTT-3') and was controlled by a PCR amplification of *β*-actin, using the following primers (all primers from Sigma): forward 5'-GCCAACCGTGAAAAGATGAC-3' and reverse 5'-GACCAGAGGCATACAGGGACAG-3'. Primers were used at 333 nM concentration. Both PCR were performed in the same 25 µl mix (12.5 µl REdTaq Ready Mix Sigma, 8.5 µl distillated H_2O, primers, 2 µl template DNA). PCR of 35 cycles with denaturation at 94°C for 1 minute, annealing at 61°C for 1 minute and extension at 72°C for 1 minute (and with initial denaturation at 94°C for 5 minutes and final extension at 72°C for 5 minutes) was performed in GTC thermal cycler (Cleaver Scientific). Amplified DNA fragments were loaded on a 1% agarose (Sigma) gel prepared in 100 ml TBE buffer 1x with 4 µl Sybrsafe (SYBR, safe DNA gel stain 10,000x concentrated in DMSO, Invitrogen). One hour DNA electrophoresis at 85 V in TBE 1x was performed. The SMARTLADDER SF (Eurogentec) marker ladder was used to determine the length of the fragments amplified.

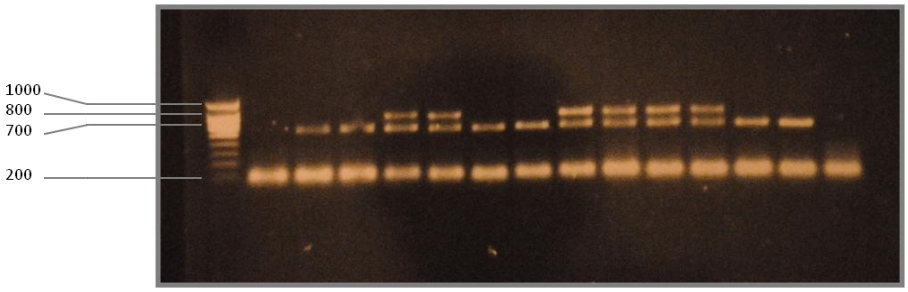


Figure 7: Example of DNA genotyping. In this figure, an example of PCR agarose gel used to discriminate transgenic RasB8 and non transgenic mice is shown. On the left of the picture, the molecular weight marker ladder is observed; band size displayed refers to base-pair (bp). The house keeping gene used is *β-actin*, corresponding to the 700 bp bands. It is present in all samples, with the exception of negative controls (first and last column), in which distillated water was added to PCR reaction mix, instead of DNA. 800 bp bands correspond to the transgene; hence, samples which show two bands are RasB8 transgenic mice. In non transgenic mice, only the *β-actin* band is visible.

2.2. FLOW CYTOMETRY

2.2.1. General principles of immunofluorescence

Immunofluorescence is a technique that permits to visualise cellular antigens by using fluorescent molecules (fluorochromes) conjugated to specific monoclonal antibodies. Fluorochromes, if excited at their excitation wavelength, are able to emit light at their emission wavelength. Two different kinds of immunofluorescence staining exist: direct and indirect. The direct immunofluorescence technique uses antibodies directly conjugated to a fluorochrome (primary antibody). The indirect technique uses one primary non-labelled antibody and a second antibody conjugated with a fluorochrome (secondary antibody). Indirect immunofluorescence can also utilise a tertiary fluorochrome-conjugated antibody if the secondary is not-labelled, or can adopt an antibody biotin-conjugated. For the latter case, streptavidin (SA)-conjugated fluorochrome is required.

Antibody	Fluorochrome	Emission peak (nm)	Clone	Company
CD4	PE	575	GK 1.5	BD Biosciences
CD4	APC/ Cy 7	774	GK 1.5	Biolegend
CD8	APC	660	53-6.7	BD Biosciences
CD8	APC/ Cy 7	774	53-6.7	BD Biosciences
Ly-6C*	SA-PerCP/Cy5.5	690	HK 1.4	Biolegend

CD11b	PE/ Cy 7	774	M1/70	BD Biosciences
B220	APC	660	RA3-6B2	BD Biosciences
CD44°	FITC	525	IM7	BD Biosciences
CD62L	PE	575	Mel-14	ImmunoKontact
INF-γ	PE/ Cy 7	774	XMG 1.2	Biolegend

Table 2: Antibodies. In the table, antibodies used in this experimental work are shown with emission peak of their conjugated fluorochromes. (*) this antibody was biotinilated. All antibodies were from rat, except (°) from mouse.

If the amount of antibody used is appropriate to saturate all antigenic sites on cells, the amount of the emission light is proportional to the number of target molecules stained. The correct antibody concentration is determinated by titration. As negative control, staining is performed with an antibody of the same isotype, but which is of irrelevant specificity. Usually, the staining with the isotype is compared to the staining with the antibody, to detect the real expression of the antigen of interest.

Isotypes	Fluorochrome	Correspondent antibody	Clone	Company
IgG2b	PE	CD4	A95-1	BD Biosciences
IgG2a	FITC	GR1	R 35-95	BD Biosciences
IgG2a	APC	B220	RTK 2758	Biolegend
IgG2a	APC/ Cy 7	CD8	10 797	BD Biosciences
IgG2c*°	SA-PerCP/Cy5.5	Ly-6C	RTK 4174	Biolegend
IgG2b	PE/ Cy 7	CD11b	A95-1	BD biosciences
IgG1	PE/ Cy 7	INF-γ	MOPC-21	BD biosciences

Table 3: Isotypes. In the table, control isotypes used in this experimental work are displayed. The correspondent antibodies are also shown. (*) this antibody was biotinilated. All antibodies were from rat, except (°) from mouse.

If intracellular staining is required, it is performed after the surface staining. For intracellular staining, cells are fixed and secretion of molecules such as interferon and chemokines is usually inhibited using monensin, an antibiotic which blocks intracellular protein transport. Importantly, cell permeabilization is required to permit antibody penetration into the cells. Antibodies have to be prepared in permeabilization buffer to ensure the cells remains permeable.

2.2.2. Principles of flow cytometry

Flow cytometry is a technique which detects several parameters of a cell population after immunofluorescence staining. In this experimental study, cells were analysed using Gallios™ (Beckman Coulter) flow cytometer and results were processed with Flowjo 7.2.5 software (Treestar Inc.) or with Kaluza Flow Cytometry analysis 1.0 software (Beckman Coulter).

Flow cytometer permits to measure individual cell fluorescence and to acquire data from thousands of cells per second. The instrument is composed of different parts: fluidics, optics, electronics and computer. Samples are injected into a sheath flow and each cell is forced to pass through different laser beams that have passed through optical filters. Once fluorochromes are excited, the emission light is direct to optical detectors (in this work, named FL). The electronic system is constituted by photomultiplier tubes that convert the incidence light into electronic pulses. The signal is finally amplified and digitized for computer analysis. Flow cytometers can be equipped with more than two lasers; consequently, several fluorochromes are used at the same time to obtain diverse cell parameters from one sample. Importantly, most fluorochromes do not have a tight emission spectrum, thus compensation is needed to correct overlaps in the emission spectra. Compensation is usually established by software programs.

Data acquired by the flow cytometer are displayed in different types of graph, which can show one or two parameters at the same time. Usually, a logarithmic scale is adopted and the unit of measure corresponds to the voltage generated in the photomultiplier tube by light emitted from excited cells. Graphs which show two parameters simultaneously are utilised to identify cell subpopulations. In particular, in dot plot graphs, each dot plot represents a single cell. To initially identify the cell subpopulation investigated and to gate one specific subset, the dot plot with forward scatter (FS) on x-axis and side scatter (SS) on y-axis is visualised. FS correlates with size of cells, SS with cellular granularity. Double-positive cell dots are situated in the upper right region of the plot, whereas double negative dots on the lower left region. Single positive for the parameter on x-axis are situated in lower right region, for the parameter on y-axis in the upper left region. Other kind of two parameter graphs can be used, such as Density, Pseudocolour, Zebra and Contour plots according to the analysis to perform. Another type of graph is histogram which measures only one parameter on x-axis and the respective count of cells on y-axis. Stainings with the same fluorochrome from the same or from different samples can be overlayed on this kind of graph.

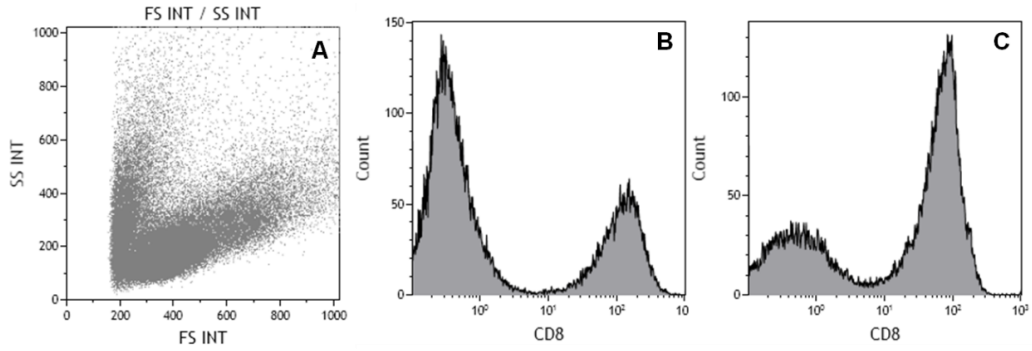


Figure 8: Dot plot and histogram. A) Example of dot plot graph in which SS and FS scatter is shown. Gating on different populations is possible, according to size and granularity. **B**) and **C**) Examples of histogram. Two distinct populations are represented in both graphs: the population with higher fluorescence is CD8 positive and it is represented by the peak on the right.

2.2.3. Surface staining protocol

Mice were killed with isoflurane inhalation. The two groups of mice sacrificed for both Carboxyfluorescein diacetate succinimidyl ester (CFSE) proliferation assay and for spleen and LN cells staining were transcardially perfused with Ringer's solution for 15 minutes. The perfusion was performed in order to remove blood cells from the brain vasculature. Brains were frozen in isopentane chilled in liquid nitrogen and kept at -80°C. For further analysis, brains can be cut at the microtome and sections analyse by immunohistochemistry at the microscope. Bilateral inguinal lymph nodes and spleen were taken. Cells from spleen and LN were dissociated by forcing through 70 µm sterile cell strainer (BD Falcon) using HBSS (Hanks Balanced Salt Solution 1x) containing 5% of complement-inactivated FCS (Fetal Calf Serum, Invitrogen). They were centrifuged at 1300 rpm for 5 minutes, the supernatant was discarded. Red blood cells in spleen were lysed with 3 ml ACK (lysing buffer), resuspended in 12 ml HBSS 10% FCS and centrifuged. LN and spleen cells were resuspended in 5 ml and in 10 ml HBSS 5% FCS respectively. Live cells were discriminated by exclusion of Trypan Blue dye and they were counted with improved Neubauer counting chamber. Cells were used for this protocol and also for the CFSE proliferation assay (section 2.3.2.). They transferred to conical 3 ml tubes (10^6 cells/ tube). They were washed with FACS® FWB (flow cytometer wash buffer), centrifuged and supernatant discarded. Cells were resuspended in 15 µl Fc-block (antibody which binds Fc-receptors, to prevent nonspecific bidings of monoclonal antibodies). Cell were then incubated at 4°C for 10 minutes. 15 µl of antibody mix was then added, cells were incubated 4°C for 20 minutes. Cells were washed with FWB, centrifuged and supernatant was discarded. 30 µl of secondary antibody

mix was added if necessary, cells were incubated 20 minutes at 4°C. Two repeated washes with FWB were finally performed. Cells were resuspended in 300 µl FWB and transferred into round-bottom tubes, read on a Gallios flow cytometer or kept at 4°C until their analysis.

2.2.4. Intracellular staining protocol

After surface staining, an intracellular staining to analyse the INF-γ production was performed in the experiment discussed in section 2.4. The BD Cytofix/Cytoperm™ Plus Fixation/Permeabilization kit was used. Supernatant was discarded and pellet was resuspended in 250 µl BD Fix/Perm solution. Cells were incubated 4°C for 20 minutes. Cells were then washed twice with 1 ml 1x BD Perm/Wash Buffer, centrifuged and supernatant was then discarded. Pellet was resuspended in 50 µl of intracellular antibody prepared in BD Perm/Wash Buffer, samples were incubated 30 minutes at 4°C. Cells were then washed twice with 1 ml 1x BD Fix/Perm solution, centrifuged and supernatant was discarded. Cells were then resuspended in 200 µl FWB and transferred into round-bottom tubes, read on a Gallios flow cytometer or kept at 4°C until their analysis.

2.2.5. Determination of cell numbers and viability by Trypan Blue exclusion

Trypan blue is a vital stain used to discriminate live cells from dead ones. Live cells with intact cell membrane are not coloured and are bright, whereas the stain traverses the damaged membranes of dead cells and they appear blue at the microscope. Trypan Blue exclusion was adopted to identify live cells and cell counting was performed using improved NeuBauer counting chamber.

2.3. CFSE PROLIFERATION ASSAY

2.3.1. General principles of the technique

CFSE has the ability to passively diffuse into cells, owing to the presence of two acetate groups that make the compound highly membrane permeable. Once inside the cell, the acetate groups are removed by intracellular esterases; the compound become fluorescent and it is trapped inside the cell owing to reduced membrane permeability. CFSE is not transferred to adjacent cells. The staining persists in daughter cells and the fluorescence is diluted at each division. Approximately 8 cell divisions can be identified before the CFSE fluorescence is too low for detection. In this experiment, proliferative ability of splenocytes from

RasB8 transgenic and non-transgenic mice was tested. Cells were labeled with CFSE and then were cultured with Concanavalin A (ConA) a T lymphocyte mitogen extracted from the jack-bean *Canavalia ensiformis*. ConA was used to non-specifically stimulate the proliferation of CFSE-labeled lymphocytes.

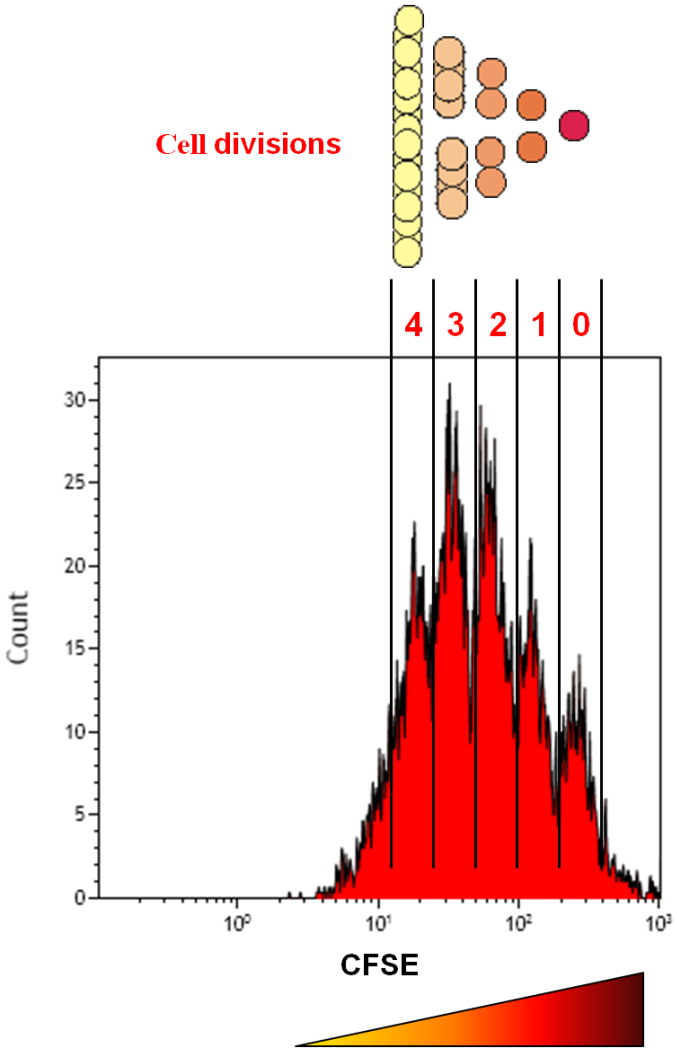


Figure 9: CFSE fluorescence is diluted at each cell division. In this figure, the principles of CFSE proliferation assay are illustrated. At each cell division, CFSE fluorescence is diluted to daughter cells. In this case, four cell divisions are shown and labeled with numbers from 0 to 4. Cells which divided more are represented in yellow in the cartoon above; in the histogram, they are represented by the less fluorescent peak.

2.3.2. CFSE proliferation assay protocol

As described in section 2.2.3, cells were counted for this experiment and for spleen and LN staining. $2x10^7$ spleen cells were harvested in a 15 ml falcon tube and washed twice with RPMI 1640 (Roswell Park Memorial Institute medium 1640 1x) without FCS. Then they were resuspended in 1 ml RPMI 0% FCS containing CFSE (Molecular Probes) 5 μM. They were incubated 10 minutes at 37°C 8% CO_2 and shaken once after 5 minutes. Then they were washed twice with RPMI 10% FCS and resuspended in 5 ml T cell medium. Then cell concentration was adjusted to $1.33x10^6$ cell/ml. Then they were plated in a 96-well plate (150 μl/ well). 50 μl of medium containing Concanavalin A (Sigma) was added to the wells to obtain two different final concentrations (2.5 μg/ml or 0.5 μg/ml). The cells were incubated at 37°C 8% CO_2 for 4 days. The cells were harvested and transferred to 96-well plates with conical wells. They were centrifuged at 1000 rpm for 5 minutes and then washed with FWB. After discarding supernatant, cell were resuspended in 15 μl Fc-block and incubated for 10 minutes at 4°C. Then 15 μl of antibody mix was added, cells were incubated 4°C for 20 minutes. The cells were washed twice with FWB, lastly resuspended in 200 μl FWB and transferred into round-bottom tubes. They were read on a Gallios™ (Beckman Coulter) flow cytometer. Every sample was analyzed in quadruplicate. CD4-PE and CD8-APC antibodies were used.

2.3.2. Data analysis

Using Flowjo 7.2.5 software, the percentages of divided T cells after ConA stimulation were counted. Cells were gated on live lymphocytes according to size and granularity. In figure 10 are shown two different representative examples of graphs used to calculate the number of divided and not divided cells.

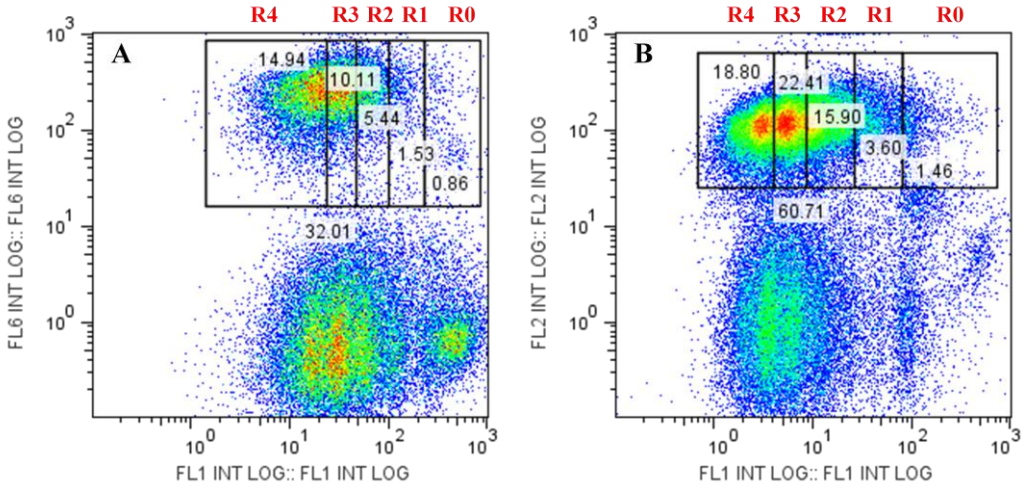


Figure 10: CFSE proliferation data analysis. Two pseudo colour graphs representative of CFSE proliferation assay data analysis. In FL1, CFSE fluorescence is displayed, whereas in FL6 and in FL2 fluorescence of CD8 and CD4 antibodies respectively are observed. In figure A and B, proliferation of CD8 and CD4 T respectively is shown after 2.5 µg/ml ConA stimulation. Each cell population was gated according to the different level of CFSE fluorescence, from R0 to R4.

On the X axis FL1 is displayed, which shows the read out of the CFSE fluorescence. FL6 and FL2, on the Y axis, correspond to CD8 and CD4 staining, respectively. Cells were gated in five different groups: R0 (not divided cells) and R1, R2, R3, R4 (divided cells). Only CD8 or CD4 positive cells were considered (upper region of the plot). The non divided cells (R0) are on the right side of graphs and have higher CFSE fluorescence. The cells which have proliferated more (R4), show decreased CFSE fluorescence (on the left side of the graphs). The percentage of divided cells was calculated as follow:

$$\%\,\text{divided cells} = \frac{\text{tot. dividing cells}}{\text{tot. cells}}$$

Where:

Total dividing cells = R1 + R2 + R3 + R4
Total cells = total dividing cells + R0

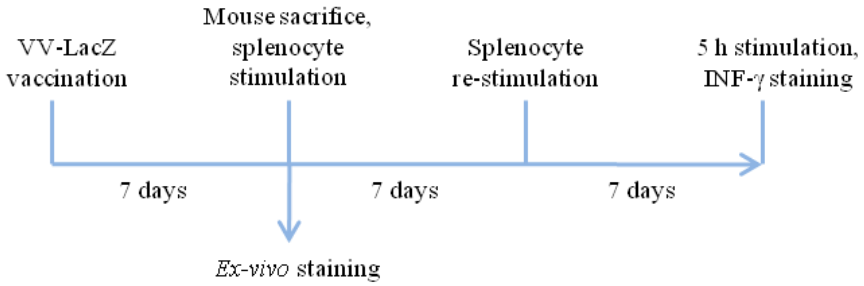
2.4. DETECTION OF SPECIFIC ANTIGEN RESPONSE

2.4.1. LacZ-recombinant vaccinia virus

Vaccinia virus (VV) was commonly used to vaccinate against smallpox (caused by variola virus) and it is a useful expression vector. In this experiment, LacZ-recombinant VV virus (VV-LacZ) (kindly provided by D. Pinschewer, University of Geneva, Switzerland) was used. LacZ is the gene which encodes for β-galactosidase (βgal), an enzyme which catalyses the hydrolysis of β-galactosides into monosaccharides. As previously explained, in the RasB8 mouse model, transformed astrocytes express this protein under GFAP promoter control (see figure 5). Thus βgal is considered an antigen specifically expressed on glial cells and potentially presented on MHC class I. In this experimental work, two βgal peptides (Biomatik, USA) were used: DAPIYTNV (βgal_{96-103}) and ICPMYARV ($\beta gal_{497-504}$), corresponding to two proposed immunodominant epitopes able to bind to the MHC class I allele H-$2K^b$.

2.4.2. Resume of the experiment

In this experiment, RasB8-129 mice were used. The aim of the experiment was to determine if a tumour Ag-specific T cell response can be detected peripherally after VV-LacZ vaccination and subsequent *in vitro* cell stimulation. The following figure clarifies the experimental procedure:



Mice were sacrificed one week after VV-LacZ vaccination, the expected peak of immunological response. *Ex-vivo* staining was performed in order to verify T cell activation; mice were selected according to the proportion of activated cells and cell growth observation performed at the microscope.

Stimulation of cells was performed with DAPIYTNV and ICPMYARV peptides. In order to confirm an immunological response against the virus, vaccinia virus peptide (VV peptide, TSYKFESV, $B8R_{20-27}$) stimulation was performed. Cells grown without antigen were used as negative control. Cells were also cultured with ConA to assess their capacity to proliferate under mitogen non-specific stimulus. For cell re-stimulation, peptides and irradiated C57BL/6 mice spleen cells were used. Irradiation was performed to prevent cell proliferation. As control, irradiated cells were plated and no proliferation was observed. For the final 6 hour of incubation, before INF-γ staining, ionomycin (iono) (Sigma) and phorbol 12-myristate 13-acetate (PMA) (Sigma) incubation was performed. Iono/PMA incubation activates lymphocytes. In particular, ionomycin is an ionophore produced by the bacterium *Streptomyces conglobatus*; it raises the intracellular levels of calcium (Ca^{2+}). PMA is diester of phorbol and activates protein kinase C (PKC).

2.4.3. Mice vaccination, sacrifice and cell culture

Mice were vaccinated intraperitoneally with $2x10^6$ plaque-forming units (PFu) of VV-LacZ. After one week, they were sacrificed by isoflurane inhalation. Spleens were taken and cells were dissociated by forcing through a 70 μm sterile cell strainer using HBSS containing 5% of complement-inactivated FCS. Cells were centrifuged at 1300 rpm for 5 minutes, the supernatant was discarded. Red blood cells were lysed for one minute with 2 ml ACK, then resuspended in 10 ml HBBS 5% FCS. Cells were then counted. 10^6 cells were harvested in one conical tube and used for *ex-vivo* staining (section 2.4.4.). For each spleen, $5x10^6$ cells/well and $2.5x10^6$ cells/well were plated in 24-well plates at five different conditions: T cell medium only (final volume of 1 ml), ConA (1 μg/ml), VV peptide (10 μg/ml), ICPMYARV peptide (10 μg/ml), DAPIYTNV peptide (10 μg/ml). To each well, 30 U/ml of IL-2 were added, in order to stimulate T cells expansion. Cells were kept on culture at 37°C, 5% CO_2 for one week. At day 4, 500 μl of medium were removed and 500 μl of fresh T cell medium supplied with 30 U/ml IL-2 were added.

2.4.4. *Ex-vivo* staining

Ex-vivo splenocyte staining was performed on day of sacrifice to determine the activation of $CD8^+$ and $CD4^+$ T cells one week after VV-LacZ vaccination, using surface staining. All procedures were performed in a P2 laboratory and cells were fixed after antibody staining using FWB fix buffer

(Flow cytometer Wash Buffer with Fixative) for the last two washes. Antibodies used were: CD4 APC/Cy7, CD8 APC, CD44 FITC and CD62L PE.

2.4.5. Cell re-stimulation

Splenocytes were re-stimulated with the three different peptides previously mentioned after one week-culture. Re-stimulation was performed using C57BL/6 spleen cells. C57BL/6 mice were sacrificed; spleen cells were taken and counted as previously described. The cell number required in each well for re-stimulation was $5x10^6$ cells. Cells were centrifuged and divided in three 15 ml falcon tubes according to the cell number required. Then they were resuspended in 10 ml T cell medium. VV, ICPMYARV, DAPIYTNV peptides were added to the tubes at a final concentration of 10 μg/ml. Cells were then incubated for 90 minutes at 37°C 5% CO_2 and then irradiated at 100 Gy. Cells were then washed twice with HBSS medium 5% FCS. They were finally re-suspended at the required volume in 90 U/ml IL-2 CTL medium. 500 μl of medium was removed from each culture well and 500 μl of prepared cell suspension for re-stimulation was then added to each well. Fresh 90 U/ml IL-2 CTL medium was added to wells not stimulated with peptides.

2.4.6. INF-γ staining

Seven days after re-stimulation, cells underwent 5-hour peptide or iono/PMA stimulation before intracellular INF-γ staining. Wells were carefully washed twice and cells were collected and re-suspended in T cell medium. Cells which were stimulated with peptides were split in two different tubes to performed peptide or iono/PMA stimulation. Cells stimulated with ConA were not analysed, but their growth was observed at the microscope as a positive control. Cells were centrifuged at 1500 rpm for 6 minutes and supernatant was discarded. 1 ml of T cell medium containing different peptides at the established concentrations was then added; in iono/PMA stimulation tubes ionomycin was added at 1 μg/ml and PMA at 100 ng/ml. Cells were incubated for 1 hour at 37°C 5% CO_2. BD Golgi Stop™ was then added to each sample (1:1000 diluition) and then 4 hour incubation was performed. After incubation, surface staining was performed using the following antibodies: CD8 APC, CD4 PE and CD44 FITC. Then INF-γ intracellular staining (INF-γ PE/ Cy7) and isotype (IgG1 PE /Cy7) control staining were performed.

2.5. STATISTICAL ANALYSIS

Statistical analyses were performed using Sigma STAT® software. *P-values* were considered statistically significant if < 0.05.

3. RESULTS

In this experimental work, we investigated the peripheral immune status of RasB8 astrocytoma mouse model, functionally and phenotypically. We analysed *in vitro* responsiveness of T cells to mitogen and we performed surface staining of splenocytes and LN cells. Furthermore, we analysed peripheral responsiveness to a tumour-associated antigen. This chapter is divided according to the two different experimental approaches.

3.1. SPLENOCYTES AND LN ANALYSIS

In this experiment, we used RasB8-CD1 mice. We tested two different groups of mice with their corresponding control groups. The first group was constituted by six 4-6 week-old (wo), asymptomatic transgenic mice whereas six non-transgenic, littermate mice composed the control group. The second group was formed of six ill, older mice (> 12 weeks old) and its control group by six non-transgenic, age-matched mice. We performed surface staining of splenocytes and LN cells and CFSE proliferation assay.

3.1.1. Surface staining of splenocytes and LN cells

We sacrificed mice and we took spleen and inguinal lymph nodes. We isolated cells and then we lysed red blood cells from spleen. After that, we performed surface staining to analyse leucocyte subpopulations, using the following markers: Gr-1, CD4, Ly-6C, CD11b, B220 and CD8.

3.1.2. Quantitative analysis of T and B cells in spleen and LN

We calculated percentages of B cells ($B220^+$), CD4 T cells and CD8 T cells related to the total live leucocytes in spleen and in lymph nodes. We also calculated the ratio of CD4/CD8 T cells. With respect to B cells (figure 11A), there were always higher cell percentages in spleen than in LN (in spleen the mean of all groups is 42.4 ± 5, in LN 12 ± 2). No significant differences were observed between groups. CD4 T cells (figure 11C) are more represent in LN than in spleen. A significant difference was observed only in LN, between ill RasB8 mice and littermate group. As for CD4 T cells, CD8 T cells (figure 11B) are presented at higher percentages in LN. No significant differences were observed between groups for both organs. The ratio CD4/CD8 (figure 12) is significantly

different between ill RasB8 mice and littermates in LN. In spleen, the corresponding percentages follow the same trend.

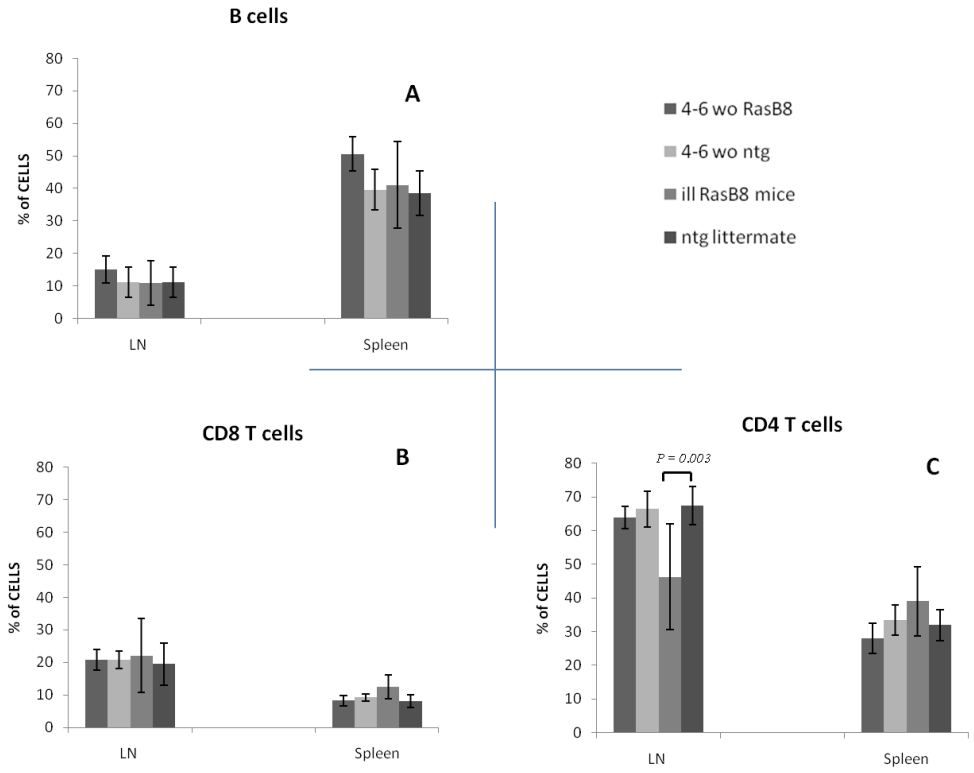


Figure 11: CD4 T cells are significantly diminished in ill RasB8 mice LN. Spleens and LNs were taken from the indicated groups. Cells were prepared from individual mice; they were surface stained with antibody to B220 (**A**), antibody to CD8 (**B**) and antibody to CD4 (**C**) and analysed by flow cytometry. Cell percentages in the leucocyte gate are shown. Group means ± standard deviation (SD) are displayed. *P-value* was calculated with unpaired Student *t-test*.

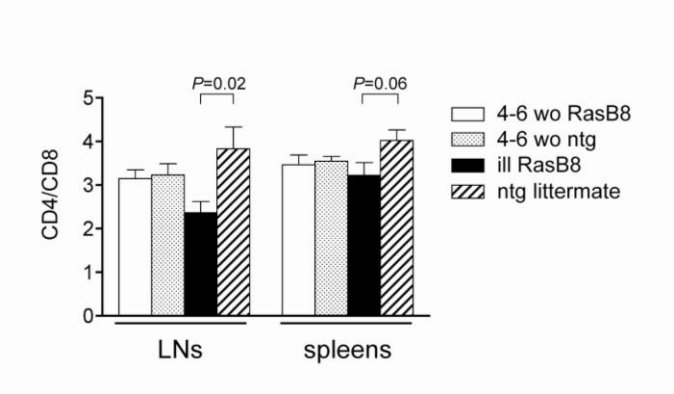


Figure 12: LN cells of ill RasB8 mice have a decreased CD4/CD8 ratio. Proportions of CD4 and CD8 cells in lymphoid tissues and spleens of the indicate groups of mice were obtained from flow cytometry analysis and ratios were calculated. Bars indicate means ± SD of mouse groups. *P-value* was calculated using an unpaired Student *t-test.*

3.1.3. Memory CD4 and CD8 T cells: Ly-6C marker

We analysed the expression of the Ly-6C marker, proposed to be associated with memory function, in CD4 and CD8 T cells. We gated cells according to their positive expression of CD4 or CD8, then we detected subpopulations expressing high level of the memory marker using histograms. We analysed cells from both LN and spleen. With respect to $CD4^+$ cells in the LN, two distinctive cell subpopulations which differently express Ly-6C were not observed in the majority of samples (data not shown). For this reason, no graph showing LN $CD4^+$ Ly-$6C^{high}$ cells are displayed in this section. In all graphs, 100% is equal to the total number of cells in $CD4^+$ or $CD8^+$ population.

There were no significant differences between groups for $CD4^+$ Ly-$6C^{high}$ cells (figure 13). Concerning $CD8^+$ cells, there was a trend towards higher Ly-6C expression on $CD8^+$ cells in older mice, but this was not statistically significant (figure 14).

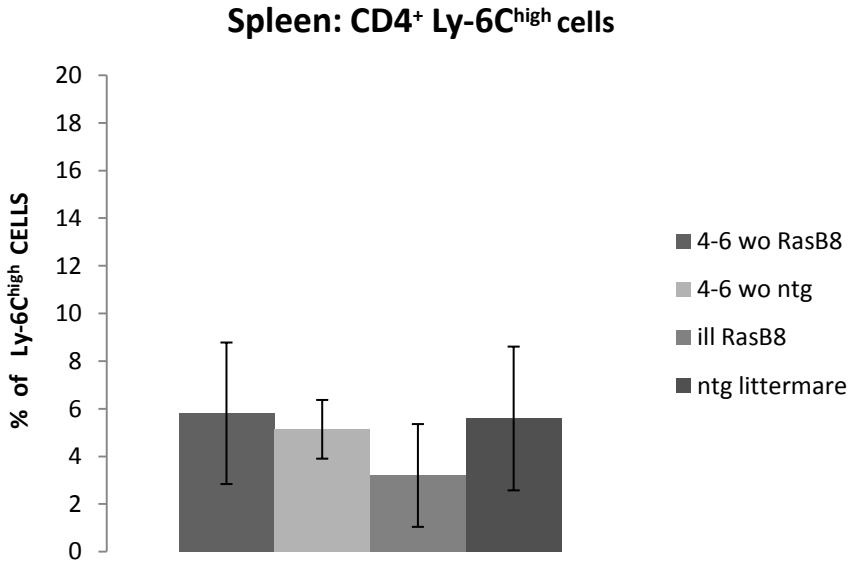


Figure 13: No differences in $CD4^+$ Ly-$6C^{high}$ splenocyte percentages between RasB8 and control groups. Cell percentages were obtained from flow cytometry analysis; lymphocytes were gated according to positive expression of CD4. CD4 T cells from LN are not displayed because a CD4 positive subpopulation expressing high level of Ly-6C was not detected in all samples. Means of different groups ± SD are displayed.

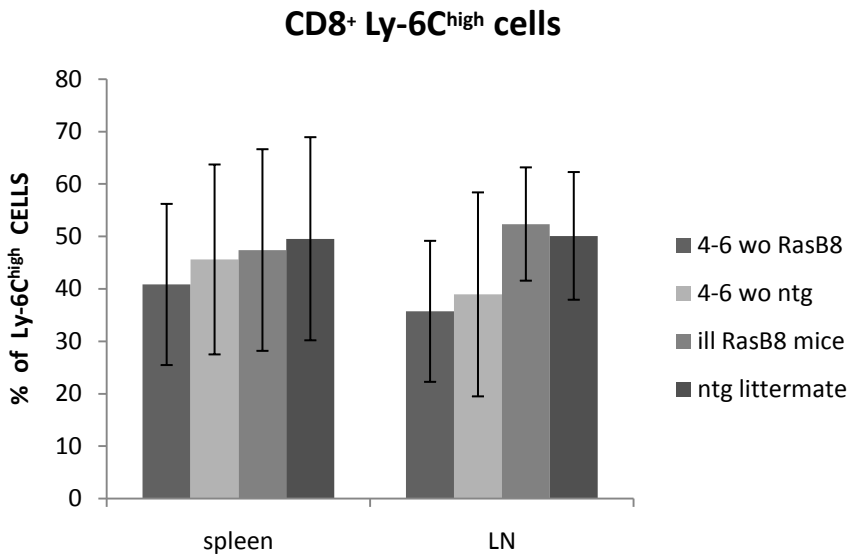


Figure 14: A trend towards higher percentages of CD8$^+$ Ly-6C^{high} cells is observed in older rather than in younger mice. Means ± SD of mouse groups are shown. A flow cytometry analysis was performed; splenocytes and LN cells were gated according to their CD8 positive expression.

3.1.4. Myeloid-derived suppressor cells

The MDSCs are a group of myeloid cells including precursors of macrophages, granulocytes, dendritic cells and myeloid cells at earlier stages of differentiation (Youn et al., 2008).

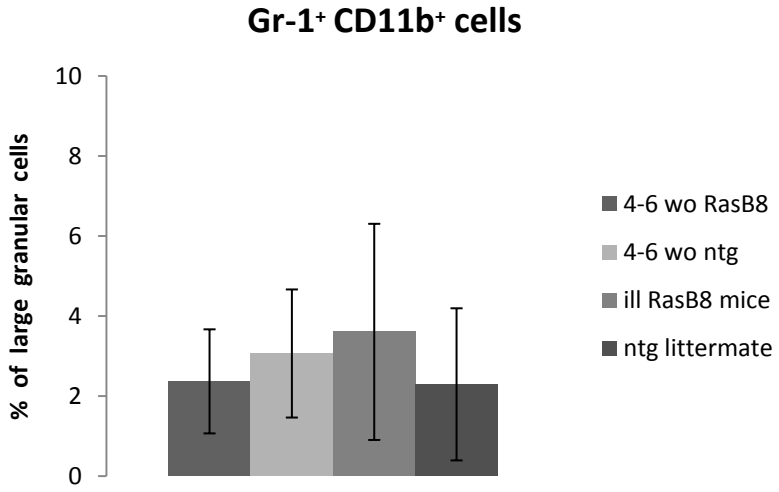


Figure 15: Myeloid-derived suppressor cells are detected in all mice. Gr-1$^+$ CD11b$^+$ cells (MDSCs) were identified by flow cytometry of surface stained splenocytes. Percentages were calculated in the total large granular cell (FCS^{high} SSC^{high}) gate. Means of defined groups are displayed; error bars represent SD.

MDSCs are able to suppress T cell responses and circulating MDSCs are found to be increased in cancer patients. In mice, they are generally defined as Gr-1^+ $CD11b^+$ cells (Youn et al., 2008). We detected Gr-1^+ $CD11b^+$ splenocytes (figure 15) but there were no significant differences between tumour-bearing, ill and control mice.

3.1.5. CFSE dilution assay for *in vitro* T cells proliferative response

To determine the proliferative capability of peripheral T cells, we used a CFSE proliferation assay. Lymphocytes that were employed for spleen and LN staining were also utilised in this test. We labelled the cells with CFSE, a fluorescent compound which is incorporated in cells and is diluted in daughter cells at each cell division (see section 2.3.1.), and we stimulated the CFSE-labelled cells *in vitro* for 4 days with the polyclonal mitogen ConA. We tested two different concentrations of the mitogen. At day 5, we harvested the cells and stained them for CD4 and CD8. Percentages of dividing cells were calculated as explained in section 2.3.3.

3.1.6. Results of proliferation assay

We analysed both CD4 and CD8 T cell response to ConA at low (0.5 µg/ml) and high (2.5 µg/ml) concentration.

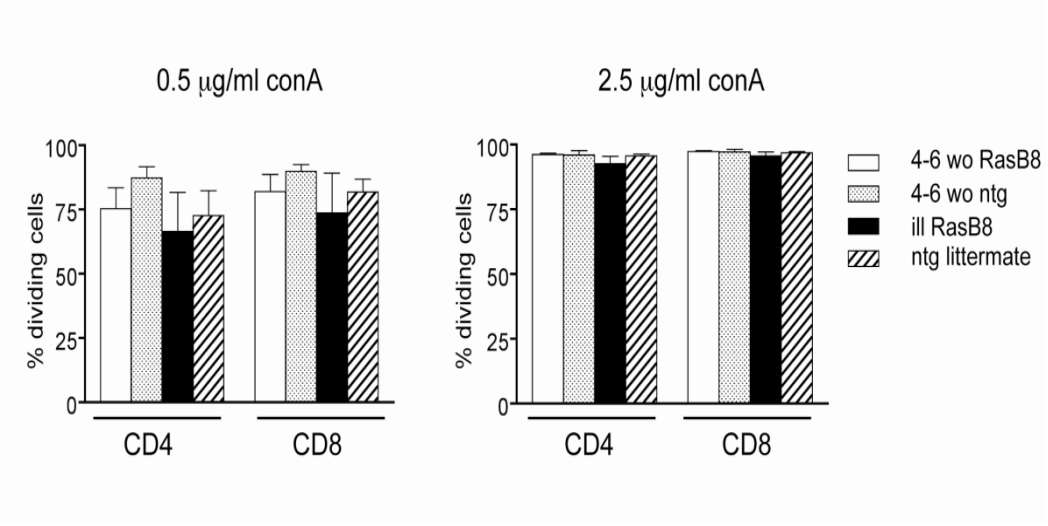


Figure 16: ConA dose-dependent proliferation of CD4 and CD8 T cells. Spleen cells from indicated mouse groups were CFSE labelled and stimulated *in vitro* for 4 days with low (0.5 µg/ml) and high (2.5 µg/ml) concentration of the mitogen ConA. Dividing cell percentages were calculated as explained in section 2.3.3. Means SD are shown.

We measured the responsiveness of splenocytes as percentage of dividing cells of either the CD4 or CD8 gated populations (figure 16). We observed that between 60% and 91% of cells from individual mice proliferated with low dose ConA, and more than 95% of cells proliferated with high dose ConA. We did not found significant differences between mouse groups.

3.2. *IN VIVO* PRIMED T CELL IMMUNE RESPONSE

In this experiment, we used RasB8-129 mice to asses *in vivo* T cell function. The specific immune response was analysed at the peripheral level, in particular after *in vitro* re-stimulation of mouse splenocytes. We infected mice with live recombinant VV-LacZ. This procedure can vaccinate mice against antigens encoded in the viral sequence (VV, Lac-Z).

Mice were divided into three different groups: transgenic (TG), non transgenic (NTG) and control. The latter was formed by two non transgenic non-vaccinated mice. Both transgenic and non transgenic mice groups were composed of six mice; four of them were selected for the last analysis according to the immune cell activation status and according to the cell growth observed *in vitro*.

3.2.1. LacZ Vaccinia Virus vaccination and mouse sacrifice

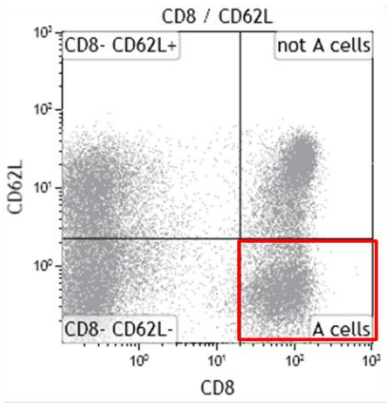
We vaccinated transgenic and non transgenic mice with VV-LacZ virus by intraperitoneal injection. Mice were sacrificed seven days after vaccination. We utilised non vaccinated mice as control.

3.2.2. *Ex-vivo* phenotype

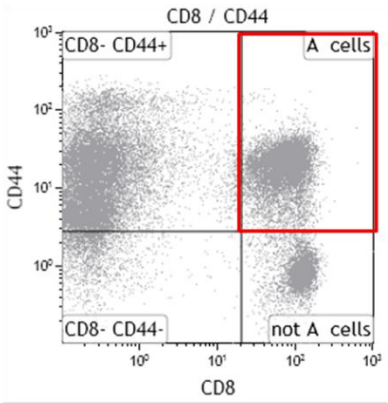
T cell activation status was investigated to assess whether there was significant T cell stimulation by the vaccination. Splenocytes were stained *ex-vivo* 7 days after vaccination, the predicted peak of the antiviral immune response. We assessed activation by high expression of CD44 and low expression of CD62L. In fact, in activated cells CD44 is highly expressed whereas the expression of CD62L is low. We observed differently activated cell populations (figure 17). In vaccinated mice, the percentage of activated cells is higher ($CD62L^{low}$ $CD8^{+}$ and $CD44^{high}$ $CD8^{+}$ in NTG mouse, $CD44^{high}$ $CD8^{+}$ in TG mouse) or comparable ($CD62L^{low}$ $CD8^{+}$ in TG mouse) to the percentage of non-activated cells. Conversely, in non vaccinated mice, we observe that non-activated cell percentage is greater than activated cell percentage. Although we cannot measure the

activation status of splenocytes of the same mice before vaccination, all vaccinated mice had higher proportions of activated CD8 T cells than the non-vaccinated controls.

TG vaccinated mouse

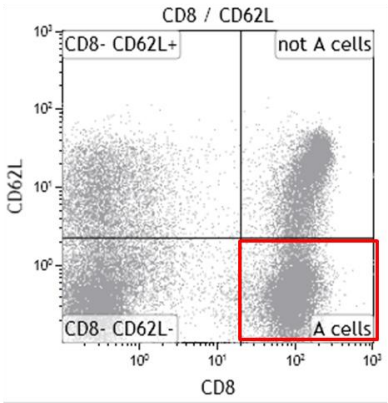


Gate	%Gated
All	100,0
A cells	11,4
CD8- CD62L-	39,9
CD8- CD62L+	36,2
not A cells	12,4

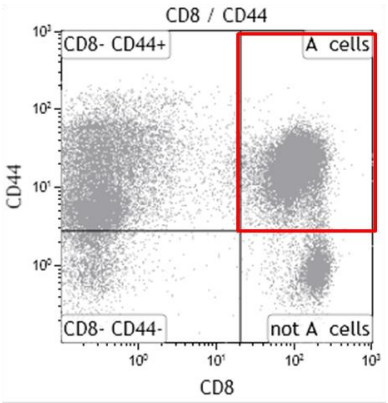


Gate	%Gated
All	100,0
A cells	16,5
CD8- CD44-	13,8
CD8- CD44+	62,3
not A cells	7,3

NTG vaccinated mouse



Gate	%Gated
All	100,0
A cells	24,3
CD8- CD62L-	37,7
CD8- CD62L+	23,0
not A cells	14,9



Gate	%Gated
All	100,0
A cells	33,5
CD8- CD44-	8,2
CD8- CD44+	52,5
not A cells	5,7

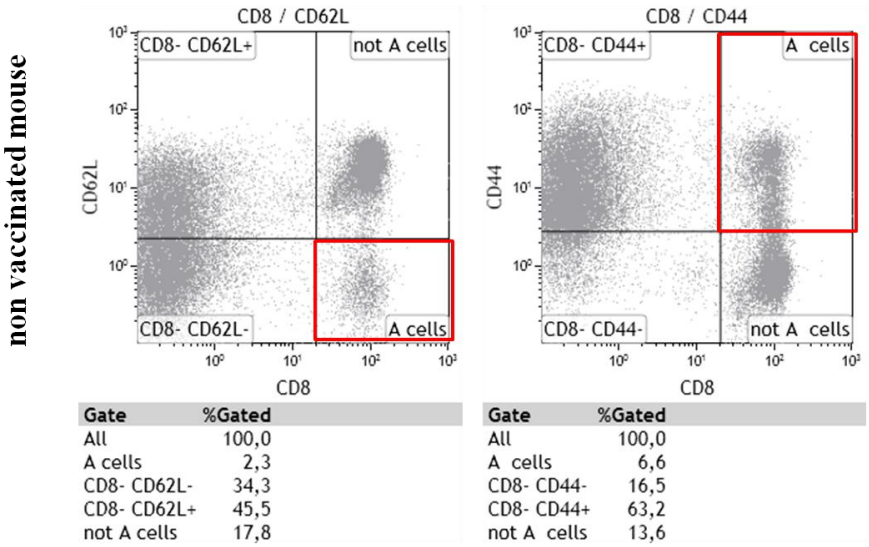


Figure 17: Differently activated CD8 T cell subpopulations after VV vaccination. Activation status of CD8 T cells was tested 7 days after recombinant VV vaccination in transgenic and non transgenic RasB8 mice. Different dot plots show representative examples of *ex-vivo* splenocyte staining. Cell activation was determined using CD44 and CD62L markers. Activated CD8 T cell subpopulations are highlighted with red rectangle. Non vaccinated mice were used as negative control.

Furthermore, we used the absolute number of cells to compare activated cells from different mouse groups. The absolute number was calculated referring to the amount of cells counted before the surface staining. We gated the cells according to the positive expression of CD4 or CD8; then we obtained proportions of these cells which were $CD62L^{low}$ and $CD44^{high}$. In figure 18, the number of activated CD8 T cells is shown. There was a high standard deviation above all for NTG mouse group. We observed a statistically significant difference only between the TG and the control group; the high SD in the NTG mouse group may influence the lack of statistical power for CD8 activated cells. We focused our attention on $CD8^+$ cells rather than $CD4^+$ cells because the former is the principle cellular type involved in the specific response we investigated. Moreover, we did not obtain statistically significant differences in the percentages of activated CD4 cells between groups (data not shown).

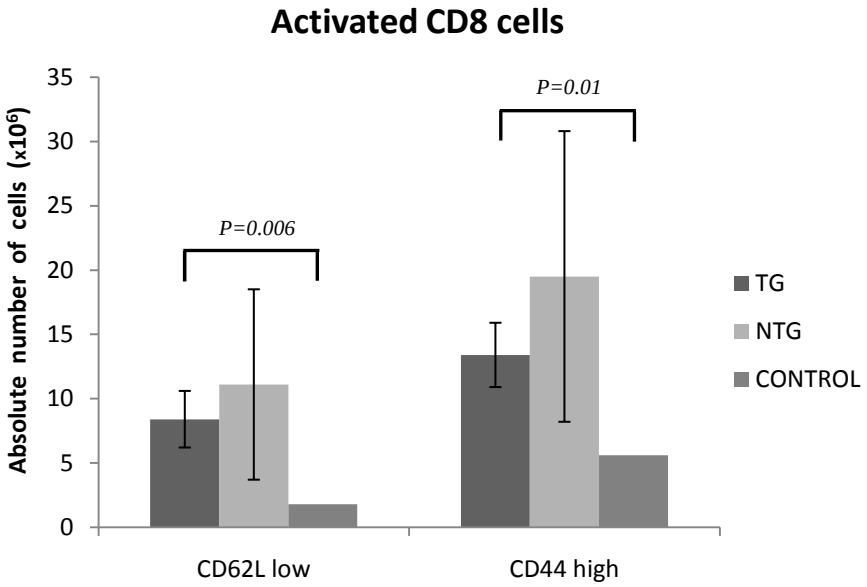


Figure 18: Significant augmentation of activated CD8 T cell in vaccinated mice. Transgenic and non transgenic RasB8 mice were vaccinated with recombinant VV virus; CD8 T cell activation status was tested 7 days after vaccination (immune response peak). Activated CD8 T cell number was determined by flow cytometry analysis. *P-value* was calculated with an unpaired Student *t-test*. Group means ± SD are shown.

3.2.3. *In vitro* re-stimulation

We cultured mouse splenocytes and re-stimulated them *in vitro*. For re-stimulation, diverse peptides were tested: a viral peptide sequence (VV peptide) and two different βgal peptides (ICPMYARV and DAPIYTNV), from the protein encoded by the LacZ gene. To define cell growth, we used cell culture medium in negative control wells and ConA in positive control wells. To facilitate T cell growth, IL-2 was added in each well. After 7 days culture, re-stimulation was performed using irradiated C57BL/6 splenocytes previously incubated with different peptides. Cells cultured with ConA did not undergo re-stimulation with irradiated splenocytes. In negative control cell wells, we utilised irradiated splenocytes (without specific peptide) for cell re-stimulation.

3.2.4. Selection of spleens for INF-γ production analysis

In this experiment, six transgenic and six non transgenic RasB8-129 mice were sacrificed. Four mice per group were selected for INF-γ staining, according to the percentages of *ex-vivo* stained activated CD8 T cells and according to the capacity of splenocytes to grow *in vitro* after stimulation and re-stimulation (table 4).

	Mice	Number of cells / spleen (x 10^6)	% $CD62^{low}$ cells *in* $CD8^+$	% $CD44^{high}$ cells in $CD8^+$	*In vitro* growth (day 6)	*In vitro* growth (day 11)
1	TG	59	28	65	++	+
2	TG	72	31	51	++	+
3	TG	92	26	36	+	-
4	TG	70	42	65	+	+/-
5	TG	62	46	70	+/-	+/-
6	TG	81	49	72	+/-	+/-
7	NTG	93	34	77	+/-	+
8	NTG	114	54	85	+	+/-
9	NTG	75	33	54	+/-	+/-
10	NTG	90	33	68	+/-	+/-
11	NTG	54	41	67	+	-
12	NTG	70	44	72	+/-	+/-
13	control	92	12	41	+/-	+/-
14	control	49	11	29	+/-	+/-

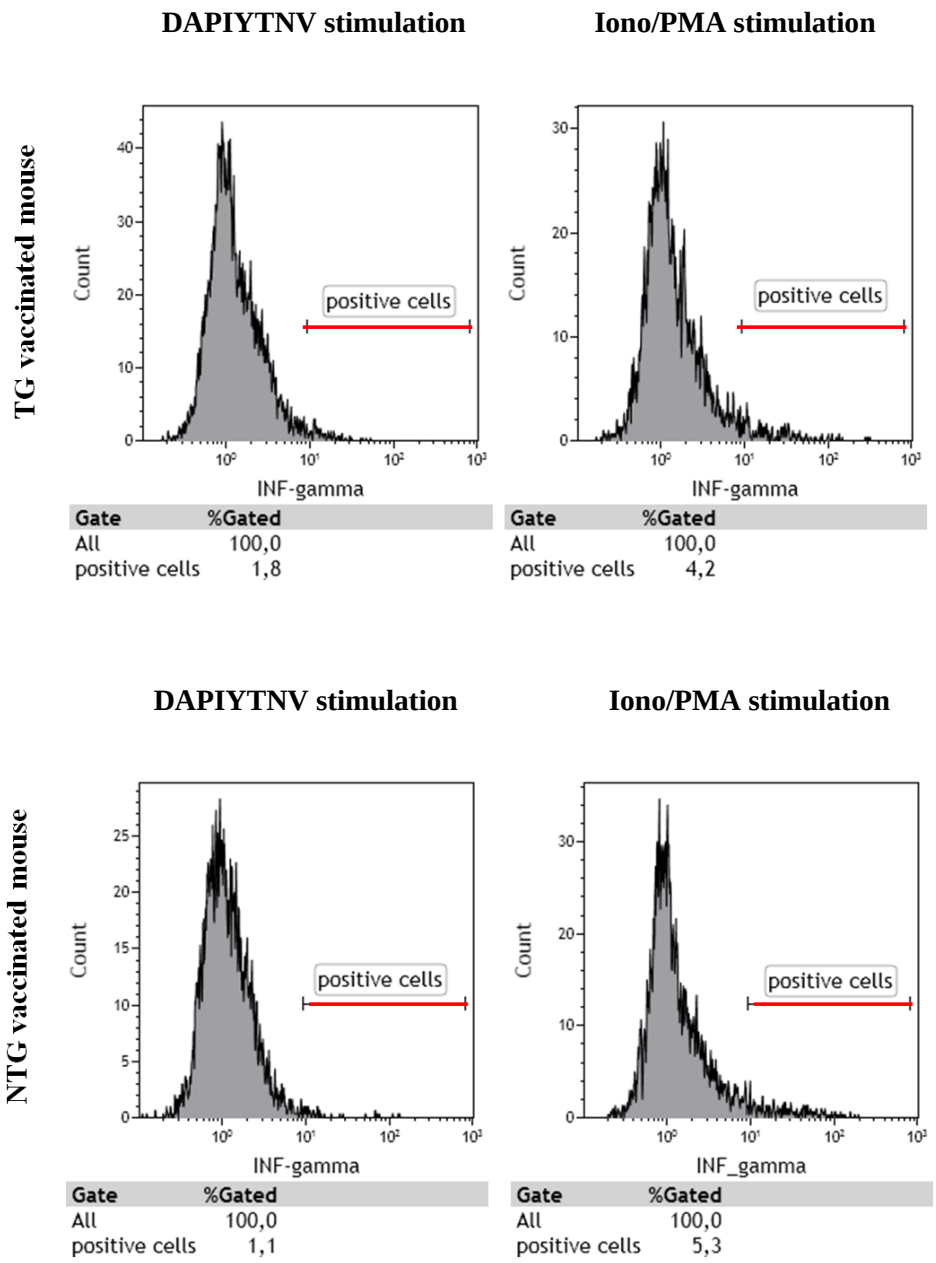
Table 4: Selection of activated splenocytes for INF-γ production analysis. Transgenic and non transgenic RasB8 mice were vaccinated with Lac-VV and sacrificed after 7 days. Spleen cells were isolated and stimulated *in vitro*. Splenocytes were selected for INF-γ production analysis according to CD8 T cell activation status and to growth *in vitro* of VV peptide-stimulated cells. Cell growth was evaluated as follows: (++) number of lymphocytes higher than in negative control, activated lymphocytes; (+) number of lymphocytes comparable to the control, only few cells activated; (+/-) number of cells comparable to cells in control wells, no activated lymphocytes; (-) lower amount of cells than in control wells. Selected mice are displayed in red; the cell absolute number is based on the cell count performed before the *ex-vivo* surface staining.

A vigorous anti-viral immune response may result in major clonal expansion of lymphocytes in the spleen. We therefore preferentially selected spleens with high cell yield for further analysis. Both CD44 and CD62L markers were considered in the selection. With respect to cell growth *in vitro*, there was no clear correlation of cell number or activation status (scored as *in vitro* growth in table 4) with the different peptide stimulations. The transgenic and non transgenic selected mice are visible in red in table 4.

3.2.5. INF-γ production

After *in vitro* βgal peptides stimulation, we re-stimulated splenocytes with the two respective βgal peptides; then we performed the intracellular INF-γ staining. Cells from non vaccinated mice were used as negative control. Re-stimulation with ionomycin and PMA was also performed in order to prove the lymphocyte ability to become activated and to secret INF-γ.

In figure 19, different representative histograms of INF-γ staining are shown. Only DAPIYTNV stimulated cells are illustrated as example. Increased INF-γ production is noticed in the transgenic mouse, although also in NTG mouse a percentage of INF-γ positive cells is observed. In non vaccinated mice, we do not notice INF-γ production after DAPIYTNV stimulation. We observe that iono/PMA stimulation induced more response in non vaccinated mouse than in vaccinated ones.



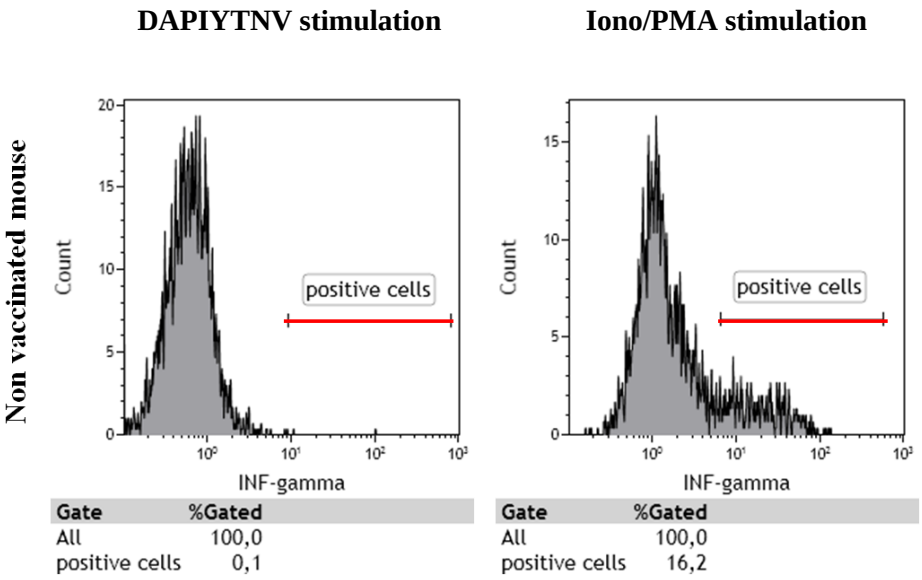


Figure 19: INF-γ production after peptide stimulation is observed only in vaccinated mice. RasB8 mice were vaccinated with VV-LacZ and isolated splenocytes were stimulated *in vitro*. Selected $CD8^+$ $CD44^{high}$ splenocytes from transgenic, non transgenic and control (non vaccinated) mice were analysed after DAPIYTNV and iono/PMA re-stimulation for INF-γ production. Illustrative histograms obtained with flow cytometry analysis are shown. Only the antibody staining is illustrated. INF-γ positive cells (red line) are displayed.

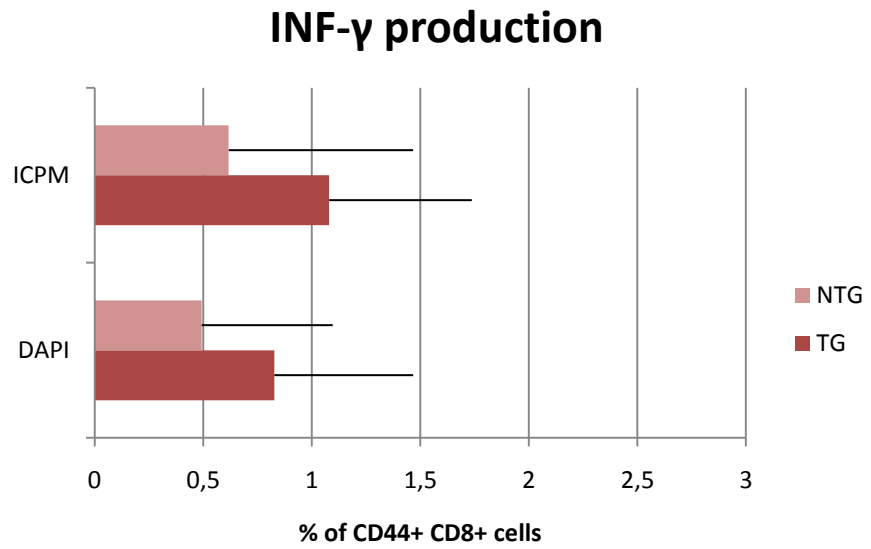


Figure 20: Trend towards higher INF-γ production in transgenic rather than non transgenic mice after peptide stimulation. INF-γ production was measured in stimulated splenocytes from both RasB8 transgenic and non transgenic mice. Lymphocytes were gated and $CD8^+$ $CD44^{high}$ stained cells were analysed with flow cytometer. Percentages were calculated subtracting INF-γ isotype staining values. Means of different groups (n=4) ± SD are shown.

The two different βgal peptides we used for splenocyte stimulation to INF-γ production are DAPIYTNV and ICPMYARV. INF-γ production was measured in $CD8^+$ $CD44^{high}$ cells from both transgenic and non transgenic mice (figure 20). A modest trend to have more production of INF-γ in transgenic mice is noticed, although no significant differences were calculated between groups. Overall, the percentages of positive cells that we obtained are at the limit of detection for both transgenic and non transgenic mice.

4. DISCUSSION

4.1. PERIPHERAL IMMUNE BALANCE IN GLIOMA

Defects in immune cellular function of glioma patients have been documented, demonstrating that a solid tumour arising in brain is able to influence peripheral immune functions through soluble factors (Fecci et al., 2006; Dix et al., 1999). In glioma patients there is evidence of cutaneous anergy to common bacterial antigens, T cell hyporesponsiveness and lymphopenia (Morford et al., 1997). With respect to a quantitative analysis of different types of cells, it has been demonstrated that relative number of peripheral blood CD4 and CD8 T cells in patients is shifted from normal: CD4 T cells are greatly diminished in glioma-bearing individuals (Waziri, 2010).

The RasB8 astrocytoma model was used in this study, to investigate the balance of different types of leucocytes at the peripheral level, in particular in spleen and in inguinal lymph nodes.

4.1.1. Glioma does not have a major impact on B cell proportions

A study on the percentage and on the absolute number of B cells in blood, showed similarities between glioma patients and healthy donors. Furthermore, immune defects in glioma patients were demonstrated to involved T cell subsets rather than B cells (reviewed by Dix et al., 1999).

Our data showed no significant differences in B cell proportions between RasB8 and control mouse groups, in both spleen and LN (figure 11 A). B cell proportions in transgenic ill mice are characterised by the highest standard deviation; this may be due to the different disease status of the mice that are only crudely graded as “ill”.

Overall, from the literature and our study, B cells do not appear to be a major target of glioma-mediated immunosuppression. However, to fully exclude this, we could also need to examine the bone marrow as another major site for B cells, and antibody titres as indicator of B cell function.

4.1.2. The proportion of CD4 T cells can be reduced in RasB8 glioma

As previously discussed, it is demonstrated that peripheral blood CD4 T cells in glioma patients are diminished in comparison to healthy donors and, consequently, the ratio CD4/CD8 T cell is altered (Waziri, 2010).

Indeed, with respect to CD4 T cell proportion, we observed a significant difference in LN T cells of ill RasB8 mice in comparison to control group (P= 0.003), demonstrating the fact that CD4 T cells proportion is diminished in old, symptomatic mice. However, in younger, asymptomatic mice, no significant differences were found. Differently to LN, in spleen CD4 T cell proportion is not significantly altered between groups.

Concerning CD4/CD8 T cell ratio, we observed a significant difference (P= 0.02) between ill RasB8 mice and littermate group in LN; in spleen, the same trend is observed (P= 0.06), although this is not statistically significant. To explain the differences observed between lymph nodes and spleen cell proportions, it can be hypothesized that in ill mice the lymphocyte recirculation can be perturbed due to the impaired muscle tone, essential for efficient lymphocyte recirculation through lymphatics. Ill mice are in fact visibly weak and their capacity of movement is limited. Indeed, lymphocyte trafficking from lymph nodes depends up on the lymphatics and requires muscular movement and tonicity. In contrast, lymphocytes enter and exit spleen by means of venous circulation, which may be preserved even in ill mice.

Overall, significant differences between CD4 T cell proportions are shown only if mice exhibit symptoms and are at the latest stages of the disease. We did not observe alterations in younger mice. In conclusion, RasB8 mice immune balance is unpaired only at later stages of the disease, differently to local immune balance, altered from early stages (Tran Thang et al., 2010). Moreover, alteration in T cell proportion is manifested within the CD4 T cell compartment rather than CD8, comparable to what is described in human glioma (reviewed in Fecci et al., 2006).

4.1.3. Memory phenotype T cells remain intact in RasB8 glioma

One of the most relevant characteristics of adaptive immunity is the capacity to provide long-term immunological memory (Parham, The immune system, 2009). Memory cells play an important role in immune responses; they rapidly respond to antigen and have an extended lifespan (Tokoyoda et al., 2009).

To investigate if glioma influences memory T cell proportion, we analysed Ly-6C expression in RasB8 $CD4^+$ and $CD8^+$ spleen and LN T cells. Ly-6C belongs to the Ly-6C family of surface glycoproteins and it is a glycosylphosphatidylinositol-anchored molecule. Ly-6C is expressed on peripheral T cells in mice bone marrow and spleen after immunization (Tokoyoda et al., 2009), suggesting that Ly-6C might be associated with differentiation and activation of T cells. Indeed, Ly-6C is considered a memory marker of CD8 T

cells (Walunas et al., 1995) and of CD4 T cells (Tokoyoda et al., 2009); in both cases it is expressed at high level.

With respect to CD4 T cells, there were no significant differences between groups. Furthermore, in lymph nodes the $CD4^{+}$ Ly-$6C^{high}$ T cell subpopulation was not detected (data not shown). Our data are in line with previous studies, in which memory CD4 T lymphocytes were demonstrated to be represented by a higher number in spleen than in lymph nodes (Tokoyoda et al., 2009).

Additionally, we observed that memory phenotype CD8 T cells are not statistically altered in transgenic mice, both in spleen and LN. However, to completely investigate proportions of this cell subset, we would also need to utilise the activation marker CD44. In fact, it was demonstrated that Ly-6C is expressed on previously activated cells and its expression is acquired only after CD44 expression (Walunas et al., 2005).

In conclusion, we demonstrated that glioma does not impact memory T cell proportions. However, to perform a complete study on memory cells it would be also interesting to analyse bone marrow cells, since it is well-established that many memory T cells reside in that tissue.

4.1.4. Myeloid-derived suppressor cell proportion is not increased in RasB8 glioma

Diverse mechanisms are implicated in glioma immune suppression; an important role is played by immunosuppressive cells: myeloid-derived suppressor cells (MDSCs), regulatory T cells and mesenchymal stem cells (Walker et al., 2009). Circulating MDSCs were found to be significantly increased in patients bearing different cancers of all stages, relative to healthy volunteers (reviewed by Youn et al., 2008). MDSCs suppress T cell responses in a specific or non-specific manner (reviewed by Youn et al., 2008).

We investigated if tumour induces a peripheral increase in RasB8 spleen MDSCs: we did not observe statistically significant differences between glioma-bearing mice and control group. Our results are discordant with another study in which increased MDSC proportions in spleen were observed in ten different mice tumour models (Youn et al., 2008), however in this study brain tumours were not examined.

In conclusion, it cannot be state that MDSCs play an important role in glioma peripheral immunosuprression. Moreover, at the local level the contribution of MDSCs to the brain tumour infiltrate was not significant in most mice (Tran Thang et al., 2010).

4.2. T CELLS DO NOT EXHIBIT IMPAIRED *IN VITRO* FUNCTIONALITY IN RASB GLIOMA

It is well-established that patients with glioma exhibit T cell hyporesponsiveness (reviewed in Morford et al., 1997). Indeed, *in vitro* studies showed that peripheral blood lymphocytes obtained from glioma patients proliferated poorly in response to mitogen or antigen stimulation (reviewed by Dix et al., 1999). Defects of T cell responsiveness may depend on defects in early transmembrane signalling (Morford et al., 1997) and on T_{reg} activity (Fecci et al., 2006).

We performed splenocyte Concanavalin A *in vitro* stimulation to determine if T cells in RasB8 mice are hyporesponsive *in vitro*, as in patients. We observed that the T cell response is mitogen dose-dependent. There were no significant differences between dividing T cell proportions in different mouse groups, even in the CD4 subset in which T cell hyporesponsiveness was observed in patients (reviewed in Fecci et al., 2006).

It is noteworthy that we performed an *in vitro* test: we indeed measured T cell responsiveness once cells were removed from the *in vivo* environment. This study did not reproduce immunosuppressive molecules and soluble factors which *in vivo* may influence cell proliferative capacity. Importantly, a correlation between the presence of the tumour and T-cell abnormalities has been discussed (reviewed by Waziri, 2010).

Overall, these data suggest that glioma peripheral T lymphocytes do not show defective proliferation capability *in vitro*. However, the lack of influence by the immunosuppressive environment has to be considered.

4.3. ACTIVATED T CELLS DO NOT RESPOND TO GLIOMA ANTIGEN

It is demonstrated that glioma patient T cells exhibit anergy *in vitro* (Morford et al., 1997). Furthermore, diverse peripheral immune defects suggest that immune responses at the peripheral level are impaired rather than functional (reviewed in Dix et al., 1999).

We investigated T cell capacity of activation and peripheral tumour-antigen specific immune response in RasB8 mice. In order to enhance or elicit a peripheral immune response, we performed a recombinant virus-mediated vaccination, using VV-LacZ. It encoded the β-galactosidase gene, which is also present in the mouse transgenic construct. Effects of this immonuotherapeutic approach were previously tested at the local level in RasB8 mice: after

vaccination, T cells infiltrated more the brains of transgenic mice and had enhanced capacity to express INF-γ (Tran Thang et al., 2010).

We observed that T cell *in vivo* capacity of activation is not affected in transgenic mice. To determine the efficacy of the VV vaccination, we investigated the activation status of CD8 T cells. There were significant differences between transgenic vaccinated and non transgenic non vaccinated mice. Non transgenic mice also show an activated T cell phenotype, but there was no statistically difference with transgenic mice. However, we did not use aged-matched mice and high variability (due to diverse immune conditions of individual mice) may have limited the sensitivity of our test to detect minor defects..

With respect to antigen specific immune response, we measured the expression of INF-γ in β-galactosidase peptide stimulated transgenic and non transgenic RasB8 splenocytes. We tested $CD8^+$ $CD44^+$ T cells only, in order to accurately identify the cell population completely activated and specific for the peptide of interest. We obtained positive stained cells, but this was at the limit of detection, in both transgenic and non transgenic mice. There were no significant differences between groups, although a trend to have more INF-γ production was observed in transgenic mice.

Overall, a specific immune response was not observed in RasB8 transgenic mice, although the capacity of T cells to become activated is comparable to control mice. Two different aspects have to be taken in consideration to consider these results. *i*) The specific immune response elicited by the recombinant VV backbone compared to that directed against the inserted gene was demonstrated to be 20-fold greater (Harrington et al., 2002). However, the response to the foreign gene is coordinately regulated with response to the vector. It can be hypothesised that the greater immune response elicited specifically against virus backbone epitopes might have prevented the boosting of the specific response for the inserted gene. Indeed, although we did not measure INF-γ stimulated by VV peptides in these experiments, a parallel study of brain infiltrating T cells did detect VV-specific T cells that had infiltrated the brain of transgenic mice (Tran Thang et al., 2010). It could be interesting to adopt another approach for mouse vaccination in order to determine if VV vaccination is appropriate to elicit both a VV and a βgal specific response, or if peripheral tolerance to the tumour associated antigens is present in RasB8 mice. *ii*) We used two different βgal peptides; both of them can be hypothesised to be insufficiently immunogenic to elicit a strong specific response. To verify their immunogenicity, it would be interesting to analyse specific immune response to other different βgal epitopes.

In conclusion, although RasB8 T cells are not able to exhibit functional βgal specific immune response after recombinant virus vaccination, this may reflect an inefficient vaccine, as their capacity to become activated was unaltered.

5. CONCLUSIONS

The aim of this thesis was to analyse the peripheral immune status in transgenic RasB8 mouse astrocytoma model. We did not found a significant imbalance in spleen and LN immune cell proportions, except at terminal stages of the disease when the CD4/CD8 T cell ratio was significantly decreased. We performed a test *in vitro* to assess T cell responsiveness to mitogen and there were no differences between transgenic and control mice. We also demonstrated that transgenic mouse T cells are able to become activated after vaccination, but we did not detect a specific tumour-associated antigen response.

In conclusion, RasB8 glioma does not significantly influence RasB8 peripheral immune status, except at later stages when CD4 T cell proportion is compromised. Furthermore, there were no alterations in cell responsiveness and activation. If these results can be extrapolated to glioma patients, they may indicate that peripheral vaccination as part of an immunotherapy may be efficient if undertaken at early stages of disease development, or perhaps in patients with low grade tumours. However, anti-glioma immune function will still have to occur locally at the tumour site in the brain, and this may be a limiting factor for clinical efficacy of a single modality therapy involving vaccination.

APPENDIX

Media and buffers

ACK buffer	Lysis buffer (FULKA); NH_4Cl 8.26 g/l, $KHCO_3$ 1 g/l, EDTA 37 mg/l.
FCS	Fetal Calf Serum (GIBCO, Invitrogen); complement-inactivated (56°C for 30 minutes).
Fix/Perm Solution	fixation/permeabilization solution (BD Cytofix/Cytoperm™ Plus Fixation/Permeabilization kit).
FWB	Flow cytometer Wash Buffer (PBS 1x supplemented with sodium azide 0.05%, FCS 5%).
FWB fix	Flow cytometer Wash Buffer with Fixative (PBS 1x supplemented with BSA 0.5%, sodium azide 0.05%, formaldehyde 1%).
Golgi Stop	protein transport inihibitor containing monensin (BD Cytofix/Cytoperm™ Plus Fixation/Permeabilization kit).
HBSS medium	Hanks Balanced Salt Solution 1x (Invitrogen), containing Hepes 10mM, Penicillin 100 μg/ml, Streptomycin 100 U/ml.
Perm/Wash Buffer	stock 10x, (diluited at 1x in distillated autoclaved water for use); containing Fetal Bovine Serum and saponin (BD Cytofix/Cytoperm™ Plus Fixation/Permeabilization kit).
RPMI medium	(Invitrogen), Roswell Park Memorial Institute medium 1640 (1x).
TBE buffer	GIBCO (Invitrogen). Containing Tris 1M, Boric Acid 0.9 M, EDTA 0.01 M. Stock at 10x, diluted in distillated autoclaved water to prepare TBE 1x.

T cell medium	500 ml DMEM (Dulbecco's Modified Eagle's Medium) high glucose (4500 mg/ml) containing Glutamax and Pyruvate + FCS 6% + Hepes 10 mM + Penicillin 100 μg/ml, Streptomycin 100 U/ml, L-Glu 2 mM, L-arg 116 mg/l, Asp 36 mg/l + β-ME $2x10^{-5}$ M.

Fluorochromes

APC	Allophycocyanin
APC/ Cy 7	APC and cyanine dye Cy 7
FITC	Fluorescein isothiocyanate
PE	Phycoerythrin
PE/ Cy 7	PE and cyanine dye Cy 7
Per CP/Cy 5.5	Peridinin chlorophyll protein and cyanine dye Cy 5.5
SA	Streptavidin

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