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Targeting BARF1 for the therapeutic control of EBV-associated malignancies

Direttore della Scuola: Ch.mo Prof. Paola Zanovello

Supervisore: Dott. Antonio Rosato

Dottorando: Riccardo Turrini

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Abstract

The Epstein-Barr virus is a γ-herpesvirus that preferentially infects human B lymphocytes. It is estimated that 95% of worldwide population is infected, but usually the infection happens during childhood and is asymptomatic. Beside the association to a self-limiting lymphoproliferative disease, infectious mononucleosis, the virus is related to the development of some human cancerous forms, which are characterized by different patterns of viral latency, like Burkitt's lymphoma (BL) and some forms of gastric carcinoma (GC), Hodgkin's lymphoma (HL) and nasopharyngeal carcinoma (NPC), and, finally, PTLD, post-transplant lymphoproliferative diseases. In addition to the latency genes, it is known the expression of different non-polyadenilated RNA (EBER) and, especially in the cases of NPC and GC, the expression of a transmembrane protein belonging to the lytic cycle, BARF1.

BARF1 is a 221-aa protein with a transmembrane domain at the C-terminal. Only recently, its transforming and immortalizing roles have been demonstrated in human cells. Moreover, its extracellular domain can be cleaved and can act in a paracrin way as a growth factor on bystander cells, and it owns mitogenic activities. Nevertheless, its mitogenic and mutagenic properties are not well understood yet, but the importance of this protein in the pathways of neoplastic progression and its expression only on infected cells (or at the surface of those that can bind its secreted forms) render BARF1 an optimal target for a therapeutic approach of EBV-related tumours.

Different strategies for the treatment of EBV-related neoplasms are currently in use in clinic. Some strategies reside on the reduction of the immunosuppressive regimen, on the exploitation of gene therapy, on the use of chemotherapy or antiviral drugs, or on the approaches of immunotherapy. The use of autologous or HLA-matched cytotoxic T lymphocytes (CTL) proved to be efficient and usually devoided of side effects, especially for PTLD patients. A different aspect of immunotherapy is based on the use of monoclonal antibodies (mAb), as it was already demonstrated in different clinical settings by the use of rituximab.

In this PhD work, the generation and the *in vitro* evaluation of different monoclonal antibodies specific for BARF1 are described. Moreover, once their activity was established on cell culture *in vitro*, this approach was translated to some pre-

clinical models, using immunodeficient mice bearing EBV-positive tumours. Also in these experiments, the antibodies proved to be therapeutically efficient.

On one side, the use of mAb for the diagnosis and for the cure of malignancies is acquiring an increasing importance in the clinic, thanks to the specificity of action of these molecules and to their relative easiness of use, if compared to the cellular adoptive therapy. On the other side, although BARF1 functions and interactions with other proteins or cells are not well studied yet, it can be regarded as a promising target for EBV-related malignancies, since it is expressed in NPC and GC and owns important transforming properties, while being a lytic cycle protein.

Riassunto

Il virus di Epstein-Barr è un γ-herpesvirus che infetta preferenzialmente i linfociti B umani. Si stima che il 95% della popolazione mondiale sia infettata, ma normalmente tale infezione avviene nell'infanzia ed è asintomatica. Oltre ad essere l'agente causale di una malattia linfoproliferativa autolimitante, la mononucleosi infettiva, la presenza del virus è associata ad alcune neoplasie umane, caratterizzate da diversi *pattern* di espressione genetica. Alcune delle neoplasie EBV-associate sono il linfoma di Burkitt (BL) e alcune forme di carcinoma gastrico (GC), il linfoma di Hodgkin (HL) e il carcinoma nasofaringeo (NPC), e infine le malattie linfoproliferative post-trapianto (PTLD). Oltre ai geni di latenza, è nota l'espressione di diversi RNA non poliadenilati (EBER) e, soprattutto nei casi di NPC e di GC, l'espressione da parte delle cellule infettate di una proteina transmembrana del ciclo litico, BARF1.

BARF1 è una proteina di 221 aminoacidi, con una porzione transmembrana al C-terminale. Solo recentemente ne è stato dimostrato il ruolo trasformante ed immortalizzante in cellule umane. Inoltre, il dominio extracellulare può essere tagliato, ed è in grado di agire in modo paracrino come fattore di crescita per le cellule adiacenti, possedendo infatti attività mitogena. In generale, tuttavia, le attività mitogene e mutagene non sono state ancora completamente elucidate, ma l'importanza di questa proteina nei *pathway* di progressione neoplastica e la sua espressione unicamente nelle cellule infettate (o in quelle che ne legano la forma secreta) la rendono un ottimo candidato come bersaglio per un approccio terapeutico delle neoplasie EBV-correlate.

Esistono diversi orientamenti terapeutici nei confronti delle neoplasie EBV-relate; alcune strategie prevedono la riduzione del regime di immunosoppressione, soprattutto per il trattamento di PTLD, la somministrazione di farmaci antivirali, la terapia genica, l'uso di chemioterapici e approcci di immunoterapia. L'uso di linfociti T citotossici (CTL) autologhi o da donatori compatibili si è dimostrata efficace e generalmente priva di effetti collaterali, soprattutto in pazienti affetti da PTLD. Un altro aspetto dell'immunoterapia prevede l'utilizzo di anticorpi monoclonali (mAb), come già dimostrato in ambito clinico dall'utilizzo di rituximab.

In questo progetto di Dottorato viene descritta la generazione e la valutazione *in vitro* di diversi anticorpi monoclonali specifici per BARF1. Inoltre, una volta dimostratane l'attività su colture cellulari *in vitro*, si è traslato l'approccio ad alcuni

modelli pre-clinici sfruttando topi immunodeficienti portatori di tumore EBV-positivo. Anche in questi esperimenti è stato possibile dimostrare l'efficacia terapeutica degli anticorpi prodotti.

Da un lato, l’utilizzo di mAb sia nella diagnosi che nella cura di neoplasie sta assumendo un’importanza crescente in ambito clinico, grazie alla specificità di azione di queste molecole e alla loro relativa facilità d’uso, soprattutto se paragonati all’immunoterapia cellulare adottiva. Dall’altro, BARF1, benchè non ne siano ancora state completamente studiate le funzioni e le interconnessioni con altre molecole o cellule, è sicuramente un *target* promettente per i tumori EBV-relati, in quanto, nonostante sia una proteina espressa durante il ciclo litico, è presente soprattutto nei casi di NPC e di GC, e possiede importanti funzioni trasformanti, anche con azione paracrina.

Introduction

1 The Epstein-Barr virus

EBV is a human γ-herpesvirus that preferentially infects B cells (through the binding of CD21), but it also infects T lymphocytes, and epithelial cells.

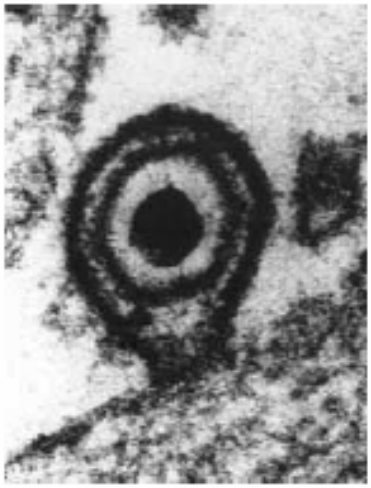

Figure 1: Electron micrograph of the Epstein–Barr virion.

The virion (fig. 1) is composed of an icosahedric nucleocapsid with 162 capsomers, and it is covered by a glycoproteic envelope. Infection happens through oral secretions and viral particles have been recovered in oropharyngeal secretions of patients with mononucleosis and, at lower titers, in EBV-positive healthy individuals. The virus preferentially infects B lymphocytes through the interaction of viral gp350/220 with host CR2/CD21 receptor. Thanks to this interaction, CD21-EBV complex is internalized through endocytosis vescicles, and then the virus can release its genome into the cytoplasm.

EBV genome is a linear double-stranded 172 Kb long DNA, which encodes for several genes. After the infection of B lymphocytes, viral DNA circularizes in an episomal form and estabilishes a latent infection. In some cases, the virus is able to reactivate the lytic cycle, which promotes the production of new viral particles and the death of the infected cells.

Viral infection has been mainly studied *in vitro* in lymphoblastoid B cells (LCL, B lymphoblasts infected *in vitro* with EBV, using B95.8 cells supernatant). LCL express the entire set of viral latent proteins, comprising 6 nuclear antigens (EBNA1, 2, 3A, 3B,

3C and LP) and 3 latent membrane proteins (LMP1, 2A and 2B). Besides the latent proteins, several small non-polyadenylated RNA are expressed (EBER), but their function is still unknown. EBNA2, EBNA3C, and LMP1 play an important role in the transformation of human B cells into LCL, thus indicating their pivotal role in the immortalization process. Nevertheless, a small fraction of LCL (about 5%) continuously enters into the lytic cycle, and expresses the viral proteins associated to this phase. Among all the lytic-phase proteins, BARF1 seems to play different mitogenic and mutagenic roles, and its expression is also present in some tumours harbouring the virus in a latent phase (like NPC and some forms of GC, (1)). Taken together, these data suggest that more attention has to be paid to the study of the expression, functions and activities of BARF1.

2 The EBV-encoded nuclear antigens

The EBV genome (fig. 2) codes for genes that affect both viral and cellular transcription. EBNA1 viral protein is expressed in every cell infected by the virus, and plays a crucial role in maintaining and replicating the viral episomal genome, thanks to the viral OriP sequence-specific binding sites. Moreover, EBNA1 interacts with different viral promoters involved in the transcriptional regulation of LMP1 and other EBNA genes. EBNA1 gene product does not seem to have a main role in B cell transformation *in vitro*; nevertheless, an oncogeneic role has been proposed for EBNA1 protein, since it induces a B cell lymphoma in mouse B cells and seems to be involved *in vitro* in Burkitt lymphoma cells (2, 3).

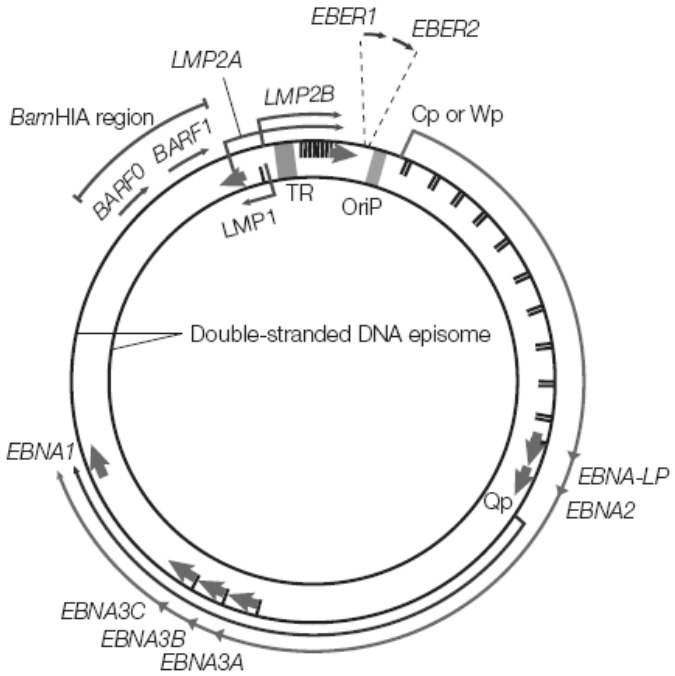

Figure 2: Diagram showing the location and transcription of the EBV latent genes on the double-stranded viral DNA episome. The latent proteins include the six nuclear antigens (EBNA 1, 2, 3A, 3B and 3C, and EBNA-LP) and the three latent membrane proteins (LMP 1, A and 2B). The highly transcribed non-polyadenylated RNA *EBER1* and *EBER2* are also represented. The locations of the BARF0 and BARF1 coding regions are also shown. Modified from Young *et al*. (4).

EBNA2 plays a pivotal role in B cell transformation process *in vitro* (5). Moreover, this protein interacts with transcriptional activators for different cellular (e.g., CD23) and viral genes (e.g., LMP1 and LMP2). EBNA-LP acts with EBNA2 and is necessary for the efficient growth of LCL. The transcriptional activation mediated by EBNA2 and EBNA-LP interaction is modulated by EBNA3 protein family: EBNA3A and EBNA3C are necessary for the *in vitro* transformation of B lymphocytes; EBNA3C can also co-operate with RAS protein and unpairs the cell cycle checkpoints (fig. 3).

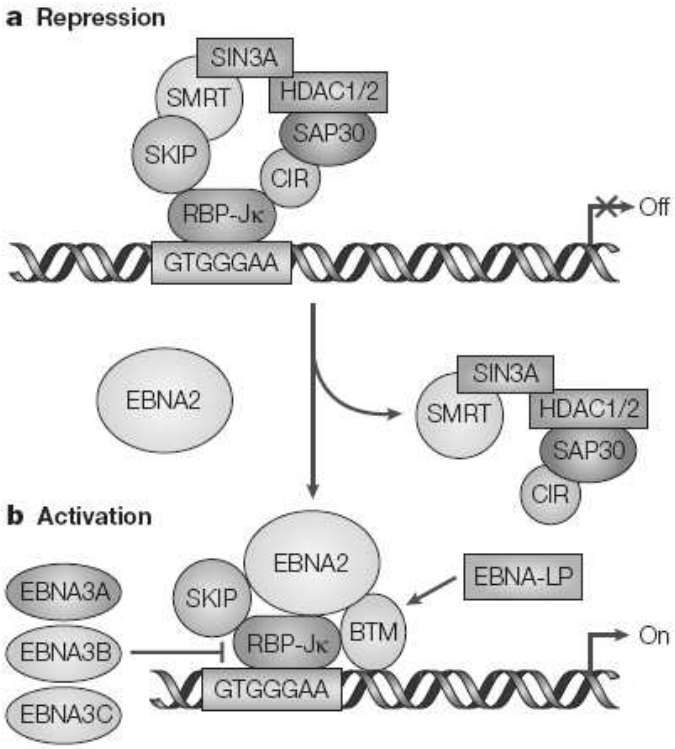

Figure 3: The EBV-encoded nuclear antigens. a) EBNA2 functions as a transcriptional activator by interacting with the DNA-binding Jκ-recombination-binding protein (RBP-Jκ) and relieving the transcriptional repression that is mediated by a large multiprotein complex consisting of SMAT, SIN3A, histone deacetylase 1 (HDAC1) and HDAC2. b) EBNA-LP cooperates with EBNA2 in RBP-Jκ-mediated transcriptional activation by interacting with the acidic activation domain of EBNA2. The EBNA3 family of proteins modulates EBNA2-mediated RBP-Jκ activation by interacting with RBP-Jκ and competing for binding and activation by EBNA2. Modified from Young *et al*. (4).

3 The EBV-encoded latent membrane proteins

LMP1 is the main transforming protein of EBV; it functions as a classic oncogene in rodent-fibroblast transformation assays and it is essential for EBV-induced B-cell transformation *in vitro* (6). LMP1 has pleiotropic effects when it is expressed in cells, resulting in the induction of cell-surface adhesion molecules, activation of antigen, and upregulation of anti-apoptotic proteins (for example, BCL2 and A20) (7). LMP1 acts as a constitutively activated member of the tumour necrosis factor receptor (TNFR) superfamily, and activates several signalling pathways in a ligand-independent manner. Functionally, LMP1 resembles CD40 — another member of the TNFR superfamily — and can partially substitute for CD40 *in vivo*, providing both growth and differentiation signals to B cells (8) (fig. 4).

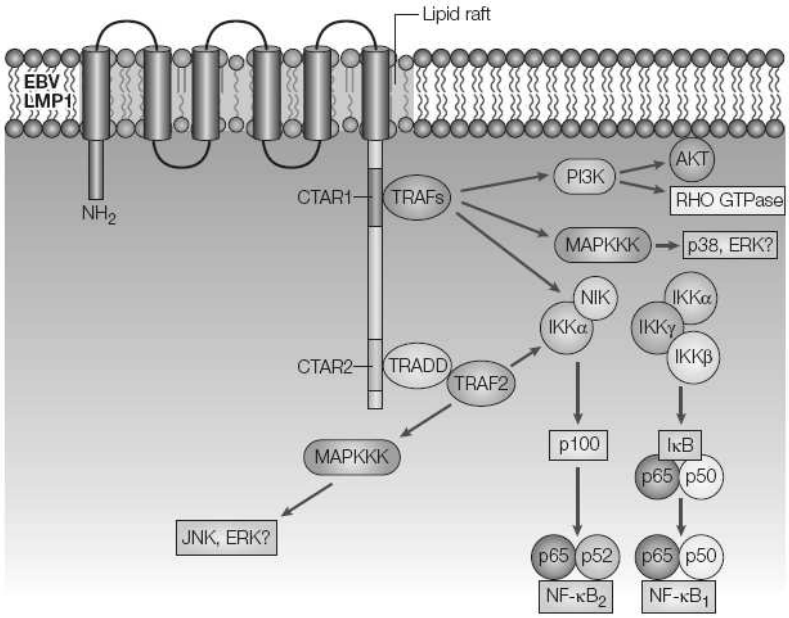

Figure 4: LMP1 is an integral membrane protein of 63 kDa and can be subdivided into three domains: first, an amino-terminal cytoplasmic tail (amino acids 1–23); second, six hydrophobic transmembrane loops, which are involved in self aggregation and oligomerization (amino acids 24–186); third, a long carboxy-terminal cytoplasmic region (amino acids 187–386), which possesses most of the signalling activity of the molecule. Two distinct functional domains referred to as C-terminal activation regions 1 and 2 (CTAR1 and CTAR2) have been identified on the basis of their ability to activate the nuclear factor-κB (NF-κB) transcription-factor pathway. Modified from Young *et al*. (4).

The LMP2 proteins, LMP2A and LMP2B, are not essential for EBV-induced B-cell transformation *in vitro* (9). However, expression of LMP2A in B cells in transgenic mice abrogates normal B-cell development, allowing immunoglobulin-negative cells to colonize peripheral lymphoid organs (10). This indicates that LMP2A can drive the proliferation and survival of B cells in the absence of signalling through the B-cell receptor (BCR). LMP2A can transform epithelial cells and enhance their adhesion and motility, effects that might be mediated by the activation of the phosphatidylinositol-3-kinase–AKT pathway (11). Repressive effects of LMP2A expression have been recently reported in human and murine B cells, and many of these target B cell-specific factors, resulting in a phenotype that is similar to that of malignant Reed Sternberg (HRS) cells in Hodgkin's lymphoma and germinal-centre B cells. In addition to these effects, LMP2A was found to induce expression of a range of genes that are involved in cell-cycle induction, inhibition of apoptosis and suppression of cell-mediated immunity.

4 Other EBV latent transcripts

Two non-coding and non-polyadenylated RNA, namely EBER1 and EBER2, are expressed in all the viral latency forms, even though they do not seem to be crucial for the transformation of B cells into LCL (5). The expression of EBER in Burkitt lymphoma cells enforces their tumourigenicity, promotes cells survival and induces the production of IL-10.

A group of abundantly expressed RNA that are encoded by the *Bam*HIA region of the EBV genome was originally identified in nasopharyngeal carcinoma (NPC), but they were subsequently found to be expressed in other EBV-associated malignancies, such as Burkitt's lymphoma, Hodgkin's lymphoma and nasal T-cell lymphoma, as well as in the peripheral blood of healthy individuals (12-14). These highly spliced transcripts are commonly referred to as either *Bam*HIA rightward transcripts (BART) or complementary-strand transcripts (CST) (15, 16). The protein products of these open reading frames remain to be conclusively identified. Another transcript that is generated from the *Bam*HIA region is BARF1, which encodes a protein that was originally identified as an early antigen expressed on induction of the EBV lytic cycle. Recent studies have shown that BARF1 is a secreted protein that is expressed as a latent protein in EBV-associated NPC and gastric carcinoma (17, 18). BARF1 shares limited homology with the human colony-stimulating factor 1 receptor (the *FMS* oncogene) and displays oncogenic activity when it is expressed in rodent fibroblasts and simian primary epithelial cells (19).

5 EBV infection in immunocompetent host

Primary EBV infection usually happens in the first decade of life of human beings, and gives as a result an asymptomatic infection. Infected hosts secrete EBV-particles in the saliva, thus permitting the oral transmission.

Primary infection in adolescence or adulthood can give raise to a self-limiting lymphoproliferative disease known as infectious mononucleosis (IM). Patients with acute IM shed high titres of infectious virions in the throat and, sometimes, in other lymphoid organs. The EBV infection leads to a strong cell-mediated immune response, which can keep the infection under control.

The virus passes through the epithelium and infects naïve B cells in the underlying tissue. Here, EBV expresses latency genes that induce B cell activation and calcium mobilization, and the viral products improve mRNA synthesis, homotypic cell adhesion, and CD23 and IL-6 expression (4).

The expression of EBV proteins induces the B cell to become blast, without any need of external signals. The transformed cell migrates to the follicle, where the viral transcription program changes, and only EBNA1, LMP1 and LMP2 are transcribed. LMP2 induces infected B cells to form a germinal centre by themselves in the mucosal follicle, where the isotypic switch takes place. LMP1 inhibits the signal that drives cells out of the germinal centre. In these ways, EBV is able to induce cells proliferation (20, 21).

When they reach the peripheral tissues, latently infected cells shut off the expression of all the viral proteins and they seem to survive as memory B cells (4). In the first phases of IM, the number of these cells in blood can reach 50% of all infected cells (fig. 5).

Once it is in the memory compartment, EBV is able to establish a long-term persistence; nevertheless, it should be able to replicate in order to invade new hosts. In case of signals inducing B cells to differentiate into antibody-secreting plasma cells, EBV can be reactivated. Since plasma cells migrate to the mucosal epithelium, these cells can release virions on the mucosal surface and in the saliva (22).

6 EBV-associated malignancies

Although the presence of EBV after the primary infection is usually asymptomatic, the virus is described to be associated to different kinds of B cell lymphomas. There are three histologically and clinically distinct types of EBV-associated B-cell lymphoma that show different patterns of latent gene expression and appear, from Ig-gene sequencing, to derive from cells at different stages of the B-cell differentiation pathway: lymphoma in immunosuppressed individuals, Burkitt's lymphoma and Hodgkin's lymphoma (fig. 5). The different stages in the B-cell differentiation pathway show the role that the microenvironment assumes in the development of B-cell EBV-associated malignancies (23). Besides B cell lymphomas, EBV is associated to some kinds of T-cell and NK-cell lymphomas, and to carcinomas,

like nasopharyngeal carcinoma and gastric carcinoma. Noteworthy, different types of EBV-associated malignancies can be immunologically grouped according to the latency patterns expressed by the virus (4).

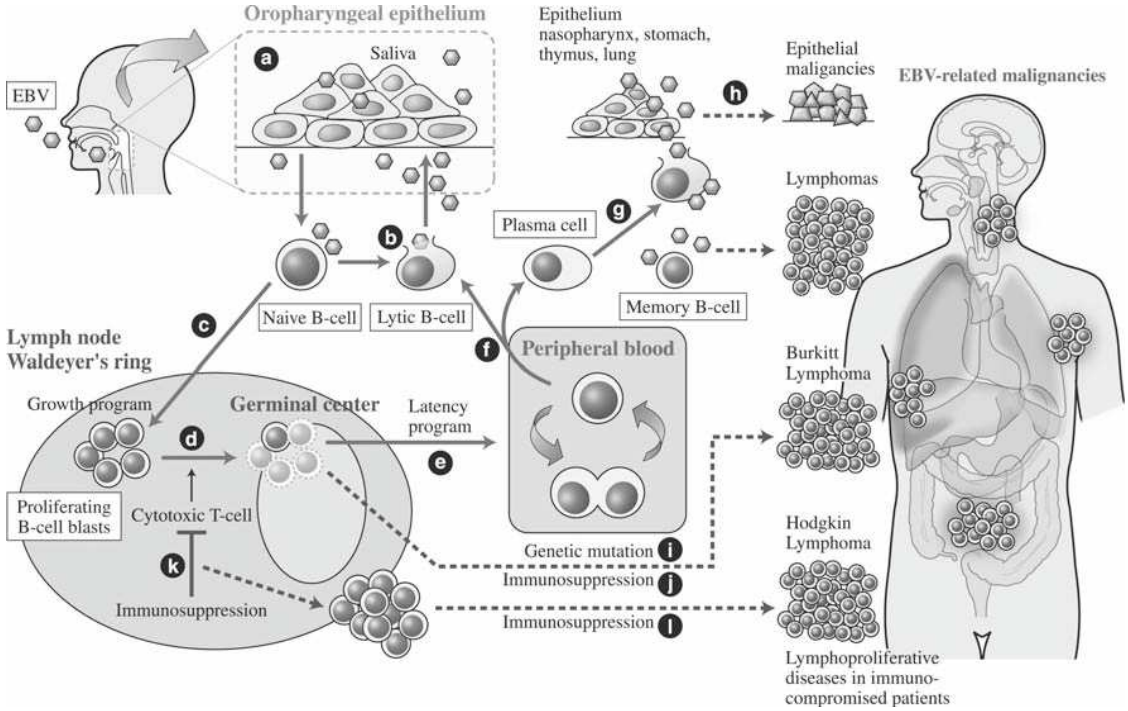

Figure 5: EBV infection and pathogenesis of EBV-related diseases. The virus primarily infects oropharyngeal epithelium or n**aïve** B cells infiltrating mucosa (a). EBV-infected B cells enter either the lytic cycle or the latent cycle. In the lytic cycle, viral particles are produced and shed into saliva, again infecting other mucosal cells and lymphocytes (b). EBV-infected B cells entering the latent cycle migrate back into the lymphoid tissue (c). There, lymphocytes enter the growth program, become blasts, and undergo proliferation. A considerable ratio of the proliferating lymphocytes is eliminated by cytotoxic T cells before and through a germinal center reaction (d). Thereafter, the infected B cells express the latency program. In this program, expression of antigen molecules that induce cytotoxic response by EBV-specific T cells is ceased, and the infected B cells become resting memory B cells (e). This way, EBV evades surveillance by the immune system, accomplishing lifelong infection in resting memory B cells. EBV-infected memory B cells persist at a frequency of 1–50/10^6 B cells in the peripheral blood and act as a long-term reservoir for the virus. Occasionally, EBV-infected memory B cells replicate the virus and release infectious viruses into saliva (f). Some EBV-infected memory B cells differentiate into plasma cells. The EBV is also released from plasma cells, entering the lytic cycle as they migrate into peripheral tissues (g). EBV released from B cells in the lytic cycle are considered to be the viral source in EBV-related epithelial neoplasms, such as nasopharyngeal and gastric carcinomas (h). EBV-associated Burkitt lymphoma is thought to occur when the germinal-centre B-cell blasts are stuck at the proliferative stage because of activated c-*myc* oncogene (i). EBV-associated Hodgkin's lymphoma is considered to arise from EBV-infected B cells blocked at the germinal centre as a result of cellular mutation (j). In the immunosuppressive state, lymphocytes that should be destroyed in the germinal centre are rescued in the absence of cytotoxic T cells (k). This circumstance is thought to give rise to lymphoproliferative diseases in immunocompromised patients (l). Modified from Maeda *et al.* (23).

The expression of viral proteins in type I latency is limited to EBNA1, EBER1, and EBER2. Type II latency is characterized by the additional expression of LMP1 and LMP2. Last, cells expressing type III latency present all the latency proteins and the viral RNA.

Lymphoma in immunosuppressed individuals: immunosuppressed patients, like transplanted patients under an immunosuppressive regimen or late-phase AIDS patients, are at high risk to develop B cell lymphoma. Most of lymphomas arise as a polyclonal or monoclonal lesion in the first year after the allo-transplant, when the immunosuppression is stronger. Almost every early onset lymphoma is EBV-positive and expresses type III latency. Both solid organ transplant (SOT) and haematopoietic allogeneic stem cells transplant (HSCT) are characterized in the host by an immunodeficient state. For SOT-receiving patients, immunosuppression is iatrogenically induced in order to prevent the organ rejection. In HSCT recipients, chemo- and radio-therapy abrogate the immune system, and the recovery is gradual, following the reconstitution of the donor immune system in the host.

The altered immunological surveillance characterizing the post-transplant period may cause the uncontrolled proliferation of EBV-infected and -transformed B cells, which could lead to a lymphoproliferative disease (PTLD). The development of the lymphoproliferative disorder is preceded by a pre-clinical phase, where viral DNA can be assessed and quantified in the peripheral blood. Viral DNA analysis could be used as a valid tool for the early diagnosis and for the application of therapeutic treatments (24). Moreover, PCR-mediated viral DNA quantification is useful for monitoring the response to the treatment, since therapeutic success is defined as the clearance of the viral DNA from the blood. Nevertheless, especially in SOT recipients, there is no strict correlation between disease and viral load in the blood.

Patients receiving bone marrow or solid organ transplant have a 20-120 times higher risk to develop EBV-related lymphoproliferative disease. The main risk factors are the grade of the immunosuppressive regimen, the EBV-negative status at the first transplant, the type of transplanted organ, and, for bone marrow transplant, the procedure used for the transplant (25).

Hodgkin's lymphoma: the first evidence that EBV might be involved in the pathogenesis of Hodgkin's lymphoma was provided by the detection of raised antibody titres to EBV antigens in HL patients when compared with other lymphoma patients, and furthermore, by the fact that these raised levels preceded the development of HL by several years. With the advent of cloned viral probes and Southern blot hybridisation methods, EBV DNA was initially detected in 20–25% of HL. *In situ* hybridisation provided the first demonstration of the existence of viral DNA in the HRS (Hodgkin Reed-Sternberg) cells (26). Subsequently, the demonstration of the abundant EBV-encoded RNA (EBER1 and EBER2) in HRS cells provided a sensitive method for detecting latent infection *in situ*. This technique is now generally accepted as the "gold standard" for the detection of latent EBV infection in clinical samples (27) (fig. 6).

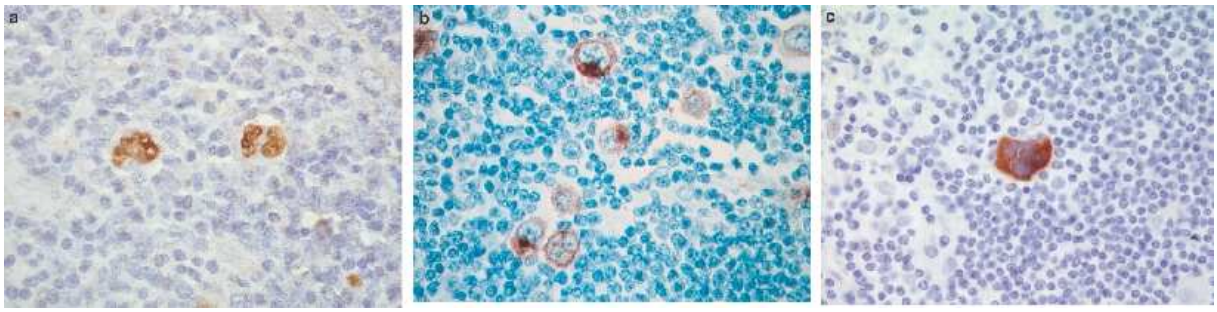

Figure 6: Gene expression in Epstein–Barr virus (EBV) associated Hodgkin's lymphoma. (a) EBER expression (brown staining) in the nuclei of Hodgkin/Reed–Sternberg (HRS) cells. *In situ* hybridisation for EBER expression is the most reliable and sensitive method to detect the presence of latent EBV infection in clinical samples. (b) The latent membrane protein-1 (LMP1) and (c) LMP2 are both highly expressed in EBV-positive HRS cells. Not shown is the consistent expression of the EBV maintenance protein, EBNA1. Modified from Young *et al.* (28).

In EBV-associated HL, viral genomes are found in monoclonal form, indicating that infection of the tumour cells occurred prior to their clonal expansion (29). In the majority of cases, EBV persists throughout the course of HL and is also found in multiple sites of HL.

The relative risk of developing HL in individuals with a history of IM, relative to those with no prior history, was shown to range between 2.0 and 5.0. It has been recently shown that the risk of EBV-positive HL is increased four-fold after IM, whereas the risk of EBV-negative HL is not increased (30).

The possibility that EBV may contribute to the pathogenesis of HL in the transformation of the progenitor cells but is subsequently lost ("hit and run"), prompted the search for evidences of defective rearranged EBV DNA in tumours that are virus negative by conventional testing (e.g., by detection of the EBER). Using fluorescence *in*

situ hybridisation (FISH) no evidence of integrated EBV genomes in EBV-negative HL was found (31). Furthermore, using quantitative PCR assays that spanned the whole genome, no evidence of deletion of EBV genomes in EBV-positive HL, or retention of EBV genomes in EBV-negative HL tissues, was found. Therefore, it seems unlikely that EBV contributes to the development of EBV-negative HL.

The rate of detection of EBV in HL depends on factors such as country of residence, histological subtype, sex, ethnicity and age. EBV-positive HL is less common in developed countries, with percentages of 20–50% for North American and European cases, 57% for HL in China, but much higher rates in underdeveloped countries (32-34). The increased incidence of EBV-positive HL in underdeveloped countries could be due to the existence of an underlying immunosuppression similar to that observed for African Burkitt's lymphoma in a malaria-infected population. This is supported by higher EBV-positive rates in HL from HIV-infected patients (35). Alternatively, the timing of EBV infection (which is likely to occur earlier in developing countries) might also be important.

Burkitt's lymphoma: first recognized because of its striking clinical and epidemiological features, the 'endemic' or high-incidence form of BL is restricted to areas of equatorial Africa and Papua New Guinea, where *Plasmodium falciparum* malaria infection is holoendemic. Conversely, sporadic BL cases occur worldwide but at a much lower frequency (at least 50-fold less). Whereas virtually every BL tumour found in high-incidence regions is EBV-positive, only about 15% of sporadic BL tumours carries the virus. Consistent features of BL tumours are chromosomal translocations involving the long arm of chromosome 8 (8q24) in the region of the *c-myc* proto-oncogene and either chromosome 14 in the region of the immunoglobulin heavy-chain gene or, less frequently, chromosomes 2 or 22 in the region of the immunoglobulin light-chain genes. This translocation results in deregulated expression of the *c-myc* oncogene. Many tumours present also genetic alterations of p53 and other defects of p53-ARF, such as many mutations in genes, which are similar to those involved in retinoblastoma. The phenotype of BL cells ($CD10^+$, $CD77^+$ and $BCL\text{-}6^+$) closely resembles that one of germinal centroblasts, both for EBV-positive and negative forms. The precise role of EBV in the pathogenesis of BL remains to be established (36). Monoclonal EBV episomes have been detected in virus-positive BL biopsies, suggesting that EBV infection precedes proliferation of precursor B-cells. The apparent

origin of BL in the germinal centre is based on phenotypic studies and is supported by the ability of BL risk factors (such as holoendemic malaria and chronic HIV infection) to stimulate B-cell proliferation in the germinal centre (because the fact that BL-cells and memory B-cells both express latency-related patterns does not necessarily imply that BL cells are derived from memory B-cells). These cells are also programmed to undergo somatic mutation of immunoglobulin genes and this event, in conjunction with the stimulation of germinal centre proliferation and EBV infection, might cause the generation and selection of B-cells carrying the *c-myc* translocation. Recent evidence also suggests that EBV might contribute to the development of EBV-negative sporadic BL by initially infecting and transforming the progenitor cell followed by virus loss ('hit and run' mechanism (28)).

Nasopharyngeal carcinoma: the tumour presenting the most stringent correlation with EBV is nasopharyngeal carcinoma. NPC is a non-lymphomatous, squamous-cell carcinoma, which occurs in the epithelial lining of the nasopharynx. The neoplasm is an uncommon disease in most countries, but it occurs with great frequency (15-30 per 100,000) in southern China, northern Africa and Alaska. EBV is consistently detected in patients with nasopharyngeal carcinoma from regions of low and high incidence. EBV-encoded RNA signal has been shown, by *in-situ* hybridisation, to be present in nearly all tumour cells, whereas EBV-encoded RNA is absent from the adjacent normal tissue. Premalignant lesions of the nasopharyngeal epithelium have also been shown to harbour EBV, which suggests that the infection occurs in the early phases of carcinogenesis (37) (fig. 7). Detection of a single form of viral DNA suggests that the tumours are clonal proliferations of a single cell that was initially infected with EBV. Viral LMP1 and LMP2 have substantial effects on cellular gene expression and cellular growth, resulting in the highly invasive, malignant growth of the carcinoma. NPC are usually subclassified, according to histologic criteria formulated by the WHO, into keratinizing squamous cell carcinoma (type I), non-keratinizing carcinoma (type II), and basaloid squamous cell carcinoma (type III) subtypes (38).

The most striking etiologic factor that non-keratinizing (types II and III) NPC have in common is the 100% association with EBV infection (39), whereas virus association with keratinizing NPCs is less consistent. Non-keratinizing NPCs can be subdivided by the expression of the viral oncogene latent membrane protein LMP1, whereas all express LMP2A (40), which has pro-survival functions, at least in B cells, and may

promote NPC metastasis (41, 42). Despite these advances, the origins of NPC remain relatively unclear. Recently, the membrane lytic protein BARF1 has been proposed as a new marker of NPC (43), and it also seems to play important mitogenic roles in NPC etiology.

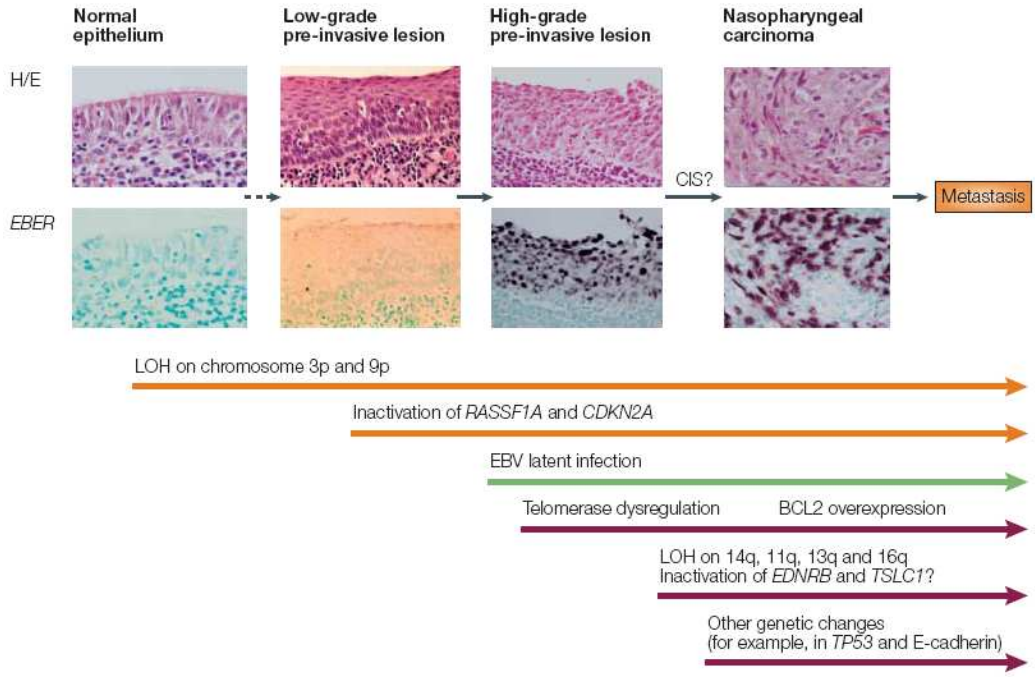

Figure 7: Proposal of the pathogenesis of NPC, possibly as a result of exposure to environmental cofactors such as dietary components (for example as salted fish). This results in low-grade pre-invasive lesions that, after additional genetic and epigenetic events, become susceptible to EBV infection. Once cells have become infected, EBV latent genes provide growth and survival benefits, resulting in the development of NPC. Additional genetic and epigenetic changes occur after EBV infection. CIS, carcinoma *in situ*; EDNRB, endothelin receptor B; H/E, staining with haematoxylin and eosin; *TSLC1*, tumour suppressor in lung cancer 1. Modified from Young *et al.* (4).

One of the major questions still surrounding undifferentiated non-keratinizing nasopharyngeal carcinoma is how the EBV-infected cells can escape the immune response: in fact, NPC cells possess normal antigen processing and are effectively recognized by EBV-specific CTL, yet they are not destroyed (44). Nonetheless, the presence of a highly suppressive tumor microenvironment and a sustained Treg activity in NPC, and poorly defined defects of the cell-mediated immune response have to be taken in consideration for understanding the NPC escape from the immune response (45, 46).

Gastric carcinoma: EBV-associated gastric carcinoma (EBVaGC) occurs worldwide, with the reported incidence varying from 5.2% to 16.0% (47). EBVaGC constitutes the largest group of EBV-associated malignancies and is now recognized as a distinct entity with distinct molecular and clinicopathological features. Lymphoepithelioma-like carcinoma (also called gastric carcinoma with lymphoid stroma) is a relatively infrequent subtype characterized by an extreme degree of lymphocyte infiltration resembling EBV-associated nasopharyngeal carcinoma. Regarding clinical features, some authors have reported that EBVaGCs tend to locate in the proximal stomach in contrast to ordinary gastric carcinomas (47).

These tumours display a restricted pattern of EBV latent-gene expression (EBER, EBNA1, LMP2A, BART and BARF1), similar to that seen in NPC. EBV-positive gastric carcinomas have distinct phenotypic and clinical characteristics compared with EBV-negative tumours, including loss of expression of p16 and improved patient survival (48). As in NPC, the precise role of EBV in the pathogenesis of gastric carcinoma remains to be determined, but the absence of EBV infection in pre-malignant gastric lesions supports the suggestion that viral infection is a relatively late event in gastric carcinogenesis (49).

Other EBV-related malignancies: the presence of EBV in different types of neoplasms has been demonstrated: 10% of gastric adenocarcinoma, and in particular the undifferentiated ones with several infiltrating lymphocytes, carries the virus (50). Recent evidences indicate that EBV has also a rare association with T-cell and natural killer (NK)-cell lymphomas (51, 52).

7 Treatment of EBV-positive malignancies

Several different approaches are available in order to treat EBV-related malignancies:

Reduction of immunosuppression regimen: after the transplant, when patients are experiencing a partial immune recovery, reducing immunosuppression alone may be successful and it has also been employed as a prophylactic strategy in recipients with increasing EBV DNA levels but no evidence of lymphoma. In solid organ transplants, where it is a previously normal immune system that has been

immunosuppressed, reduction of anti-rejection drugs may be more useful. This approach, applied in response to a rising EBV viral load, has indeed reduced the incidence of PTLD in paediatric liver transplant recipients (53). Reducing immunosuppression to restore immune responses to EBV is usually not a useful approach for treating PTLD early after HSCT since the patients are profoundly immunosuppressed and the regenerating immune system usually cannot recover fast enough to eradicate the malignant cells.

When patients are to receive chemotherapy, reduction in immunosuppression is a necessary component of treatment to minimise neutropenic sepsis. In EBV-driven PTLD, reducing immunosuppression allows host CTL function to be at least partially restored, hopefully resulting in an increase of EBV-specific CTL and elimination of virally infected lymphocytes, including those that constitute the tumour. The approach to reducing immunosuppressive therapy needs to be carefully individualised and will depend on the nature and extent of disease and the type of transplant recipient, i.e. whether they have a life supporting graft (e.g. heart) or non-life supporting graft (e.g. kidney).

The tumour response to reduction in immunosuppression is assessed by sequential monitoring of the tumour size using the most appropriate imaging modality. For EBV positive tumours, a fall in EBV viral copies in the peripheral blood may also indicate a therapeutic response (54).

Antiviral therapy: the use of antiviral drugs, like gancyclovir, may be useless to treat the cells harbouring the virus in any latency form, despite a small fraction of cells undergoing the lytic cycle. Nevertheless, it is possible to induce EBV lytic cycle using drugs or inducing kinases coded by the virus itself (like EBV timidin-kinase), which phosphorilate, and activate, gancyclovir, thus making the cells prone to respond to the treatment (55, 56). Moreover, long-term prophylaxis with antiviral agents may decrease the incidence of PTLD by limiting intercellular virus transmission; several centres use prophylactic acyclovir in the first six months after allograft, a timeframe during which the immunosuppression is more intense (57). Once PTLD is established, however, antiviral agents will have no effect on the growth of latently infected cells that are already transformed.

Gene therapy: a possible approach to EBV-related malignancies is the use of gene therapy to induce the expression of cytotoxic or inhibitory proteins specifically into

the tumour cells. For instance, viral vectors have been designed to express genes coding for cytotoxic proteins (Fas ligand) (58).

Chemotherapy: demetilating agents, like 5-azacytidine, are able to remove the repression of late lytic genes, and they are now under study in clinical trials for the treatment of NPC, Hodgkin's lymphoma and AIDS-related lymphomas (59, 60). An additional agent, 5-FU (5-fluorouracil), can promote the loss of EBV episomes *in vitro* (61).

Adoptive cell immunotherapy: the approach of adoptive immunotherapy for EBV-related malignancies relies on the knowledge of the antigenic targets, namely the viral latency proteins, which are differentially expressed in the distinct latency phenotypes (4) and almost exclusively present on malignant cells. In fact, most of these proteins are switched off in normal memory B cells. This mechanism allows the virus to escape immune control, rendering the virus-harbouring lymphocytes invisible to host and infused cytotoxic T cells (CTL). The best target for adoptive therapy against EBV malignancies is the post-transplant lymphoproliferative disease (PTLD), probably the most immunogenic among all EBV-associated tumours. In fact, early PTLD express the full spectrum of viral antigens, although more restricted patterns of viral antigen expression have been reported, particularly in late PTLD (28). Protocols for the reactivation of EBV-specific T lymphocytes used currently for adoptive immunotherapy (62) comprise several restimulations with B95.8 strain EBV-transformed lymphoblastoid cells (LCL) or APC genetically modified to overexpress discrete EBV antigens in the presence of IL-2, until a suitable number of cells is obtained for the infusion. Despite LCL express all EBV latency proteins, albeit at different density, the generated CTL are specific for several viral antigens with a well defined hierarchy of immunodominance (63) and comprise both $CD8^+$ and $CD4^+$ T lymphocytes. Data obtained thus far demonstrate that EBV-specific CTL are efficacious and safe for the treatment of EBV-positive malignancies. Interestingly, the only intent-to-treat phase-II trial (64) showed that a better outcome correlates with a higher percentage of $CD4^+$ T cells in the infusate. This observation, together with a recent work describing the complete recovery of a patient with metastatic melanoma after the infusion of a NY-ESO-1-specific $CD4^+$ T cell clone (65), opened the question whether the therapeutic successes so far described are due to a helper role or to a direct action against tumour cells by the $CD4^+$ T cells.

The first clinical results with EBV-specific cytotoxic T lymphocytes (CTL) were obtained in the setting of prophylaxis or treatment of PTLD arising after haematopoietic stem cell transplants (HSCT) or solid organ transplants (SOT). Based on such encouraging results, the same approach was then extended to other EBV-related tumours (HL and NPC) and to other virus-related diseases (like chronic active infection), which are characterized by a more restricted pattern of viral antigen expression. Comprehensive data were recently analyzed (63), and they demonstrated the efficacy of EBV-specific CTL in different settings.

Monoclonal antibodies: this approach is based on the use of antibodies against tumour antigens. Tumour-specific antibodies are able to defeat tumours not only through opsonization and phagocytosis of cancer cells by NK cells, but also thanks to the complement activation. A different strategy is the conjugation of tumour-specific antibody to toxic molecules, like radio-isotopes or anti-tumour drugs, which can selectively kill the target cells, thus limiting the side effects of the carried molecules (66).

In order to gain success with this technique, monoclonal antibodies (mAb) have to be characterized by high specificity and should be able to reach the tumour with a proper concentration. Moreover, mAb clearance has to be taken in consideration, in order to avoid side effects.

Different mAb have been generated and used for EBV-related malignancies, like anti-IL-6 mAb, responsible for cell growth, or anti-CD20, largely expressed in B cell. In particular, anti-CD20 mAb, also known as rituximab, has been largely used in EBV-related lymphoproliferations: it has been used as prophylaxis and treatment for PTLD after HSCT, with initial response rates between 55% and 100%, a range that probably reflects differences in the treated patient populations. Rituximab also has activity in PTLD after SOT, in which response rates of 44%–100% have been reported in a number of small studies (67, 68). Because CD20 expression is not confined to the malignant cells, normal B cells are also destroyed. This can be a significant concern in patients who are already immunosuppressed, and fatal viral infections have been reported after rituximab therapy (69). As rituximab can deplete B-cells for more than 6 months in these already immunosuppressed patients, it should be used as preemptive therapy for PTLD only where there is a strong probability of subsequent lymphoma. An additional concern is that when used as therapy, it does not restore the cellular immune response to EBV that is a crucial requirement if EBV-mediated B-cell proliferation is to

be controlled for long term (68). A final concern is that only one antigen is targeted and antigen-loss tumour cell variants may be selected.

8 BARF1

BARF1 gene is located at nucleotide positions 165,449 – 166,189 of the EBV genome of the strain B95.8, encoding a protein of 221 amino acids with a calculated molecular mass of 24.47 kDa (70). The surface protein BARF1 consists of two immunoglobulin (Ig)-like domains. The N-terminal domain belongs to the subfamily of variable domains whereas the C-terminal is related to a constant Ig-domain. BARF1 forms an hexamerisation structure on the cell surface, involving two principal contacts, one between the C-terminal domains and one between the N-terminal domains. The C-terminal takes contact with a large surface, which extends the beta-sandwich of the Ig-domain through the second molecule, while the N-terminal contact involves Ig-domains (71) (fig. 8). Although BARF1 is a lytic gene in EBV-infected B-lymphocytes, its expression was detected in NPC and EBV-positive gastric carcinoma tissues in the absence of the expression of other lytic genes (1), suggesting that BARF1 may be expressed as a latent gene in these carcinoma tissues.

Figure 8: Folded structure of BARF1. View of the BARF1 monomer; modified from Tarbouriech et al. (71).

This protein may play diverse functions in immunomodulation and oncogenicity, including a functional receptor for the human colony-stimulating factor (hCSF-1) (72), inhibition of interferon-alpha secretion from mononuclear cells (73), and induction of malignant transformation in Balb/c3T3 cell line (74) as well as in EBV-negative human Louckes and Akata B cell lines (74, 75). In addition, primary monkey epithelial cells were shown to be immortalised by BARF1 (76), and BARF1 was expressed in EBV-immortalised epithelial cells in the absence of the expression of LMP1 (77), which is essential for B-cell immortalization. In rodent fibroblasts, the first 54 amino acids of BARF1 were shown to be able to transform cells and activate Bcl-2 expression (19). Moreover, BARF1 could stimulate several cell protein expression: Bcl2, Myc, CD21, CD23 and CD71 (19, 74, 75). However, it is not clear yet whether such cellular genes activation is performed by intracellular BARF1 protein and/or via cell stimulation by secreted BARF1 product. The extracellular domain can be cleaved and the secreted form of BARF1 can act as a growth factor *in vivo* (78); it can inhibit alpha-interferon secretion from mononuclear cells (73) and has mitogenic activity *in vivo* (78). Potential roles of intracellular and secreted forms of the protein in these processes have to be determined and identification of BARF1 secreted protein receptor(s) now seems to be of much importance for understanding BARF1 functions *in vivo*.

Considering all the information, BARF1 gene could likely have an important role in several malignancies. However, the oncogenic mechanism induced by BARF1 is not known yet.

9 Passive immunisation with monoclonal antibodies

The treatment of cancer remains a formidable challenge owing to factors such as the difficulties in differentiating tumour cells from healthy cells to ameliorate the disease without causing intolerable toxicity to patients. Monoclonal antibodies (mAb) represent an attractive approach to tackling this problem as they can be designed to selectively target tumour cells and elicit a variety of responses once bound. These agents can directly kill cells by carrying toxic material to the target or can orchestrate the destruction of cells in other ways, such as activating immune system components, blocking receptors or sequestering growth factors.

9.1 Monoclonal antibodies generation and evolution

The total number of anticancer mAb in clinical study has risen steadily over time. In fact, the rate at which new oncology mAb entered clinical study sponsored by commercial organizations has more than tripled since the 1980s, from 4.3 per year in the 1980s to 8.3 per year in the 1990s and 13.3 per year for 2000–2005 (79). One reason for the increased investment in mAb was the evolution of the discovery technology. The initial method for producing mAb, first described in 1975 (80), involved the use of mouse-derived hybridomas. However, in humans, murine mAb were commonly (although not always) ineffective as cancer therapeutics. Studies revealed that murine candidates often had short circulating half-lives (81) and patients frequently developed antibodies to the mouse-derived proteins, which limited their utility. In addition, only certain murine mAb isotypes have been shown to effectively bind to and activate elements of the human immune system, thereby triggering cytotoxic effector functions (82). In practice, murine mAb were mostly limited to acting as targeting agents for radioactive elements or cytotoxins that might kill targeted tumour cells. Advances in genetic engineering over the years have provided numerous ways to design mAb that are more robust and efficacious compared with the original murine versions. The evolution of pure murine antibodies (e.g. OKT-3) has led to chimaeric mAb (like rituximab), antibodies with human constant regions and mouse variable regions. This technique has been central to the clinical use of antibodies. Chimaeric antibodies were less immunogenic and had the ability to trigger human effector functions and increased circulation half-life. Even though chimaeric antibodies were perceived as less foreign, and therefore less immunogenic, human antichimaeric antibody responses (HACA) have been observed (83). Further minimization of the mouse component of antibodies was achieved through complementarity-determining region (CDR) grafting. In such ‘humanized’ antibodies, only the CDR loops that are responsible for antigen binding are inserted into the human variable-domain framework. The ability to manipulate antibodies into more human-like variants finally made antibodies in the main stream of clinical use. With the isolation of genes encoding for human variable regions, their successful expression in *Escherichia coli*, and the introduction of phage-display technology along with the developments in hybridoma technology, the task of selecting fully human variable domains has been greatly simplified (84) (fig. 9).

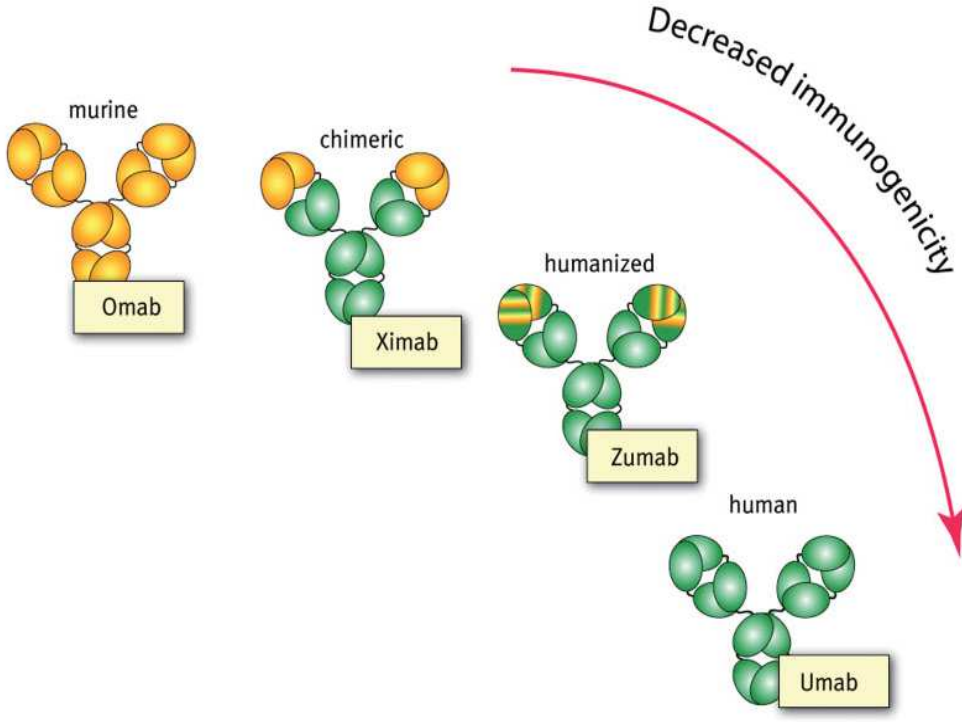

Figure 9: Classification of therapeutic antibodies in cancer. Progress in genetic engineering has facilitated the development of fully human therapeutic mAb. Original mAb technologies yielded murine (and in some cases rat) molecules. Chimaeric antibodies are genetically engineered mAb with murine variable regions (VL and VH, in yellow) and constant regions derived from a human source (in green). Humanized therapeutic mAb closely match the human germline sequence except for CDR, which are of murine (and occasionally rat) origin. Modified from Knappik et al. (84).

9.2 Therapeutic activity of monoclonal antibodies

Monoclonal antibodies are effective treatments for many malignant diseases in human beings and form one of the biggest classes of new drugs approved for the treatment of cancer in the last decade. Different antibodies have been produced and then released for the clinical use, starting from OKT-3 (murine anti-CD3, approved in 1986), and rituximab (chimaeric anti-CD20, released in 1997), to eculizumab (humanized anti-complement C5, released in 2007). One of the most famous and used mAb in clinical settings for lymphomas (and EBV-related lymphomas too) is rituximab, which binds to CD20, a cell-surface antigen expressed on almost all B-cell lymphomas and in normal B cells. In general, there are four different mechanisms of action for mAb-mediated tumour cleareance:

Antibody-Dependent Cellular Cytotoxicity: this mechanism involves binding of the antibody's Fcγ portion to Fcγ receptors expressed on immune cells with cytotoxic

capabilities such as monocytes, natural killer cells and granulocytes, which would then lead to destruction of antibody-bound cancer cells either by phagocytosis or by the release of cytotoxic granules contained in immune effector cells (fig. 10). ADCC is considered the main mechanism of action of rituximab (85).

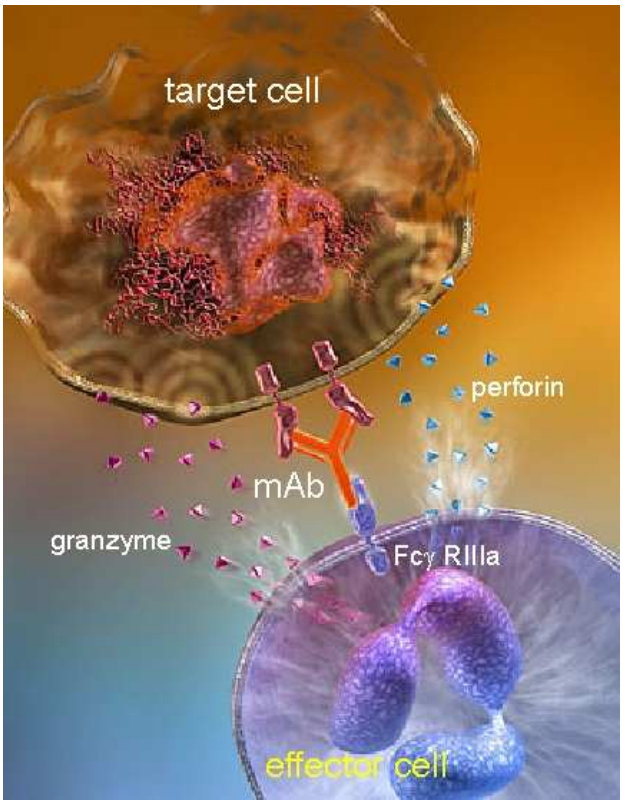

Figure 10: Proposed model for induction of adaptive immune responses by Antibody-Dependent Cellular Cytotoxicity (ADCC). An anti-tumour monoclonal antibody binds to the antigen on a tumour cell, and engages an Fcγ receptor on a killer cell. Antibody-promoted phagocytosis or antibody-direct cytolysis results in antigen processing and presentation via MHC class I or class II molecules on antigen-presenting cells. Host anti-tumour immunity is manifested by either the production of tumour-directed host cytotoxic T lymphocytes, antibodies, or both.

Complement-Dependent Cytotoxicity: given the ability of the Fc portion of some mAb to bind complement, it was proposed that they might induce cancer cell death through Complement-Dependent Cytolysis (CDC) and/or complement-dependent cellular cytotoxicity. Support for this mechanism of action was provided by *in vitro* studies demonstrating that rituximab can trigger complement-dependent killing of a variety of human lymphoma cell lines (86).

Direct induction of apoptosis: the mAb can directly bind its target on the tumour cell surface and play a role in several cellular functions such as proliferation, activation, differentiation and cell survival (87). *In vitro* studies have shown that

engagement of CD20 by rituximab triggers a cascade of intracellular signaling events and selective down-regulation of antiapoptotic factors (88).

Cross-presentation of lymphoma-derived antigens: in some cases, mAb-induced killing of malignant cells might result in priming of tumour antigen-specific T-cell responses *in vivo*. Generation of these T-cell responses might in turn be responsible for an anticancer immunity that persists far beyond the initial cytotoxic effect of the antibody itself. Supporting this mechanism, Selenko *et al* have shown that *in vitro* treatment of lymphoma cells with rituximab led to cell destruction and generation of apoptotic bodies that are taken up and processed by APC and subsequent cross-presentation of tumour-derived antigens to T cells (89).

10 Anti-BARF1 monoclonal antibody

The efficacy and safety of anticancer mAb is critically dependent on the nature of the target. An ideal tumour cell-surface target should be accessible, as well as abundant, homogeneous and consistently present on the surface of cancer cells within a tumour (90). Importantly, targets should not be highly expressed on normal cells, especially those that constitute vital organs, so that anticancer mAb can discriminate between normal and malignant cells. If ADCC or CDC modes of action are desired, then, in addition to the need for high cell-surface antigen density, the antigen–mAb complex should not be rapidly internalized by the cell because the Fc portion cannot activate relevant immune system components there. By contrast, internalization is necessary for cytotoxic activity in the case of some immunotoxins; these targets must be capable of antibody-mediated internalization, or have an intrinsically high turnover rate.

BARF1 is a surface molecule, whose expression is thought to be of significant clinical importance in some EBV-related malignancies, and mainly in NPC (91). Moreover, its secreted form can act as a growth factor and induce a paracrine loop in infected malignant cells. Nevertheless, BARF1 complete functions have not been elucidated yet, and its role in cancer transformation and progression should be further investigated. Furthermore, the early evaluation of BARF1 expression or altered expression in biopsies could be useful for the staging and treatment of patients affected by EBV-positive malignancies. In fact, the use of mAb for the diagnosis and staging of

neoplasms is currently widely used and new tools and specific antigens are always studied in order to help pathologists work. These lacks could be filled by developing a new tool for the identification *in vivo* of BARF1 on cancer cells or its secreted form on target cells.

11 *In vivo* imaging: bioluminescence

Conventional studies of cancer growth, responses to therapeutic agents, and host immune responses in small animal models rely upon euthanizing cohorts of animals at multiple time points. This experimental strategy has been the foundation for analyzing cancer pathogenesis and treatment, but there are inherent weaknesses that limit applications of this approach for testing new therapeutic compounds. Analyzing groups of mice at multiple time points after treatment precludes serial studies of disease progression in the same animal. Data from longitudinal studies in the same animal may reveal key information about animal-to-animal variations in pathogenesis or therapeutic efficacy. Furthermore, conventional animal studies require large numbers of animals to generate statistically meaningful data, which in turn necessitates larger amounts of candidate therapeutic agents for initial pre-clinical testing. *In vivo* imaging has emerged as a powerful alternative to these conventional studies of cancer pathogenesis and treatment and comprises a series of different approaches to visualize biochemical and biological events in living subjects. Importantly, these processes can be interrogated repetitively in the same animal over the course of hours to weeks, overcoming many of the limitations of conventional assays.

There are two main approaches for *in vivo* imaging: fluorescence and bioluminescence (fig. 11). In fluorescence imaging, a laser beam excitates a molecule to its excited state. The emission produced by the molecule in order to get back to its steady state is collected by the photomultiplier and analyzed. This technique can be used for the *in vivo* monitoring of drugs or the cell trafficking, but one of its limits is the choice of the fluorochrome: the emission wavelenght has to be in a precise limit, in order not to be absorbed (and then, lost) by the bystander tissue. Moreover, the analysis of green-emitting molecules (like eGFP or FITC) is not useful for the deep tissue analysis, since this light emission is not able to pass through different layers of tissue.

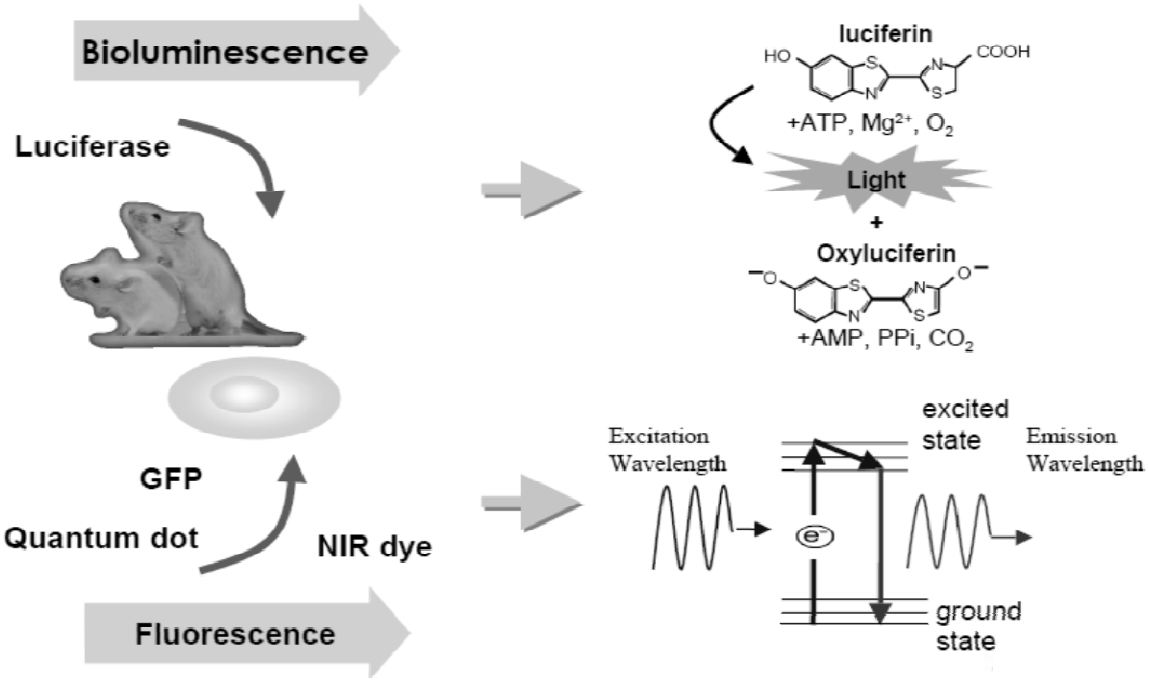

Figure 11: Exemplification of *in vivo* imaging techniques: fluorescence and bioluminescence. NIR: near infra-red; GFP: green fluorescent protein.

Bioluminescence exploits a chemical reaction where luciferin is catabolyzed into oxy-luciferin, with the concomitant production of photons. The small amount of light produced is detected by an ultra-sensitive CCD-camera. The use of this approach is wide, from drugs biodistribution, to cell trafficking and tumour growth and dissemination. The main limit is the need of the enzyme luciferase: this limitation is usually overcome with the transduction of the target cells with a vector coding for the protein. In this way, prior to analysis mice can be injected with luciferin and they can be easily monitored through time. Bioluminescence can be regarded as a potent tool for visualizing mAb-challenged tumour growth *in vivo*.

Aim of the Project

Aim of my PhD project was the generation of monoclonal antibodies specific for the EBV lytic-cycle membrane protein BARF1, and their funtional evaluation *in vitro* and *in vivo*.

To this end, in collaboration with Prof. Oriano Marin from University of Padova, we carried out a bioinformatic analysis of BARF1 protein sequence, in order to identify the best antigenic epitopes that could be used to obtain BARF1-specific mAb. In particular, three peptides were identified, synthesized and conjugated to KLH, in order to augment their immunogenicity. Several BALB/c mice were immunized, and their sera were analyzed by ELISA test and, subsequently, by flow cytometry against GRANTA-519 cells (a mantle lymphoma cell line, EBV positive). Mice with the best antibody response were sacrificed, their spleen collected and splenocytes were fused to generate mAb-secreting hybridomas. Among all the clones obtained, only those able to stain GRANTA-519 cell line were isolated and collected; then, they were grown in a special bioreactor, which allowed the expansion of cells and the collection of great amounts of antibodies. At the end of the production, immunoglobulins were purified, filtered and quantified. Later on, we characterized mAb *in vitro*: antibodies were isotypized and tested by flow cytometry against a panel of cell lines positive (GRANTA-519, C-666, EBV-positive nasopharyngeal carcinoma cell line, B65.8, marmoset lymphoma cell line producing EBV virions and BL-41 B95.8, Burkitt lymphoma cell line infected with the viral supernatant of B95.8 cell line) or negative for EBV (BL-41 and RAJI, which is EBV-positive but BARF1-negative (70)). Moreover, we performed complement-mediated (CDC) or NK-mediated (ADCC) *in vitro* cytotoxicity tests, which demonstrated the cytotoxic activity of the produced mAb. Finally, we performed passive immunotherapy experiments in SCID or $RAG^{-/-}$ γ-$chain^{-/-}$ mice bearing either EBV positive or negative tumours. All the tested clones, and in particular clone 3D4 06/08, reduced tumour growth when compared to untreated group. Noteworthy, some of the mice treated with 3D4 06/08 mAb underwent total regression of the neoplastic mass and remained completely disease-free.

Then, we exploited our *in vivo* imaging know-how and set up some experiments to precisely define the effect of the antibodies in tumour-grafted mice and to visualize their effect in a B-cell lymphoma mouse model. To this end, tumour cells were

transduced with a lentiviral vector coding for the reporter gene luciferase (92). Stably transduced cells were then used for the *in vivo* experiments as described above. For the analysis, mice were anaesthetized and injected with luciferin, the substrate of the enzime luciferase: the catabolyc reaction produced bioluminescence that can be visualized using Ivis Lumina II instrument (Xenogen).

Last, for the setting up of a B-cell lymphoma mouse model, we injected i.v. luciferase-transduced GRANTA-519 cells and analyzed the mice weekly, using the Ivis Lumina II platform.

Materials and methods

1 Cell Lines

The following human cell lines were used: GRANTA-519 are human B-cell lymphoma cells expressing EBV; C-666 is an EBV-positive nasopharyngeal carcinoma cell line; BL-41 is a Burkitt lymphoma cell line; the BL-41 B95.8 cell line is infected with the viral supernatant of B95.8 cell line, thus carrying EBV; Raji is a lymphoblastoid cell line derived from a Burkitt's lymphoma, expresses EBV but is BARF-1-deleted (70). B95.8 is a marmoset lymphoma cell line producing EBV virions. All the cell lines are cultured in RPMI 1640 medium (Euroclone), supplemented with 10% heat-inactivated Foetal Bovine Serum (FBS, Gibco), 10 mM Hepes Buffer, 1 mM Na Pyruvate, 2 mM Ultraglutamine (all from Lonza BioWhittaker), and 1% Antibiotic/antimycotic (Gibco), hereafter referred to as FBS complete medium.

NS-0 is a mouse myeloma cell line (American Type Culture Collection, ATCC), cultured in DMEM medium, supplemented with 10% heat-inactivated FBS, 10 mM Hepes Buffer, 1 mM beta-mercapto ethanol (Sigma-Aldrich), 2 mM Ultraglutamine, 1% Antibiotic/antimycotic.

2 PBMC

PBMC used in the experiments were derived from healthy donor buffy coats. Blood was diluted 1:2 in RPMI 1640 and stratified on Ficoll (GE-Healthcare). After centrifugation, PBMC were recovered. A small aliquot of cells were saved for the subsequent phenotypic analysis, while the remaining PBMC were immediately frozen at -80 ℃ in a solution containing 75% FBS and 25% Freezing solution (20% w/V D-Glucose, Fluka and 33% Dimethylsulfoxide, DMSO, Sigma-Aldrich, in RPMI 1640).

3 Phenotypic analysis

In ADCC experiments, the NK fraction of human PBMC is deputed to the lysis of cells that were previously opsonized by antibodies. In particular, NK cells bind to Fcγ and lyse the target cell without the need of MHC-I or –II interactions. Freshly obtained PBMC were analyzed in order to determine the percentage of the NK population. We

stained $2x10^5$ freshly extracted human PBMC with anti-CD16 FITC-conjugated mAb and anti-CD56 PE-conjugated mAb (both from BD Biosciences) in Staining Buffer (PBS supplemented with 3% FBS, 1% Sodium Azide, Sigma-Aldrich) for 20 minutes in ice. After incubation, cells were washed, resuspended in 250 µL PBS (Phosphate Buffered Saline, Sigma-Aldrich) and analyzed on a FACSCalibur flow cytometer (BD).

4 Epitope selection

BARF1 sequence was analyzed using bioinformatic tools in collaboration with Professor O. Marin from University of Padova. The main goal was to identify the most immunogenic peptides spanning the exposed (and secreted) protein domains. We identified 3 main epitopes, spanning all over the sequence:

$05/08_{201\text{-}221}$ CVGKNDKEEAHGVYVSGYLSQ

$06/08_{103\text{-}120}$ CRMKLGETEVTKEHLS

$08/08_{26\text{-}42}$ ERVTLTSYWRRVSL

5 KLH conjugation to BARF1 peptides

Peptides were conjugated to maleimide-activated KLH (Keyhole Limpet Hemocyanin) as a carrier protein to elicit an immune response and antibody production against the peptides.

Conjugation was performed according to Imject Maleimide Activated mcKLH Kit (Thermo Scientific). Briefly, 2 mg of each peptide were dissolved in 300 µL of Conjugation Buffer and added to 200 µL of mcKLH solution (10 mg/mL). Reaction was left for 2 hours at RT (room temperature), and then purification by desalting was performed. A desalting column was washed with 20 mL of Purification Buffer, then the conjugation mix was applied to the column and 10 aliquots of 0.5 mL of Purification Buffer were added. Each fraction was collected in a separated tube and absorbance was measured at 280 nm (using NanoDrop spectrophotometer, Thermo Scientific), to locate the fraction containing the conjugate.

The final concentration of the KLH-conjugated peptides was then evaluated by measuring the absorbance at 280 nm of a standard curve of BSA (Bovine Serum Albumin, Sigma-Aldrich) from 2 mg/ml to 0.0625 mg/ml, with 1:2 intermediate dilutions.

6 Mice immunisation

Procedures involving animals and their care were conducted according to the institutional guidelines in compliance with national laws (D.Lgs. n°116/92) and CEASA (University of Padova Ethical Committee for Animal Experiments). To produce anti-BARF1 mAb we used 3 different peptides (05/08, 06/08 and 08/08), coupled with KLH. Six-week-old female BALB/c mice were immunized by subcutaneous injections of 100 µg of each peptide emulsified in Complete Freund adjuvant (CFA, Sigma-Aldrich). Subsequent injections were performed at day 14 and 21 with 200 µL of each peptide emulsified in Incomplete Freund adjuvant (IFA, Sigma-Aldrich). When necessary, additional immunisations were performed in IFA.

7 Sera Ig titration: ELISA

Animal sera were analyzed at day 0 and 31 by ELISA test: 10 µg/ml of non-conjugated peptides were coated on a 96-well Maxisorp NUNC-immunoplate (Thermo Scientific) in 0.05 M Sodium Carbonate/Bicarbonate buffer pH 9.6 (Sigma-Aldrich), ON (overnight) at 4 ℃. The plate was washed 2 times with 250 µL/well PBS and incubated for 1 hour at RT with 100 µL/well of PBS-10% FBS. The plate was washed 6 times with 250 µL/well PBS and incubated for 1 hour at RT with sequential dilutions of mouse sera in PBS-10% FBS (starting from 1:50 to 1:6400). Negative control was PBS-10% FBS alone and sera at day 0 (1:50 in PBS-10% FBS). The plate was washed 6 times with 250 µL/well PBS and incubated for 1 hour at RT with secondary HRP-conjugated goat anti-mouse antibody (Charles River Laboratories) at the final concentration of 0.02 µg/ml in PBS-0.05% Tween (Sigma-Aldrich). The plate was washed 2 times with 250 µL/well PBS and 100 µL/well of substrate solution (prepared using Sigma-Aldrich SIGMA-Fast OPD tablets) were added for 5 minutes at RT in the dark. Reaction was stopped with 50 µL/well of HCl 3N (Carlo Erba), and the plate was analyzed at 490 nm using VictorX4 (Perkin-Elmer). Data were used to generate a titration curve.

8 Sera screening: flow cytometry

The same sera used in ELISA test were analyzed for their ability to stain GRANTA-519 cell line: $2x10^5$ cells were centrifuged and labeled with mice sera (2 µL in 50 µL of Staining Buffer), for 20 minutes in ice. Cells were then washed and labeled with secondary FITC goat anti-mouse pAb (DAKO), 2 µL in 50 µL of buffer, for 20 minutes in ice in the dark. Finally, cells were washed and resuspended in 250 µL of PBS. Cells were then analyzed using FACSCalibur flow cytometry (BD) and CellQuest software or, alternatively, FlowJo software (Tree Star).

Mice whose sera were positive by flow cytometry and gave the higher titres by ELISA were selected for hybridoma generation.

9 Hybridoma generation

Four days before fusion, mice were boosted with one additional intravenous injections of 20 µg of antigen properly diluted in PBS. The animals were then sacrificed and spleens were collected. The parenchymal structure of the spleen was mechanically destroyed and splenocytes were collected. After washing, splenocytes were counted and fused with about $25\text{-}30x10^6$ mouse myeloma NS0 cells using PEG (Hibry-Max polyethylene glycole solution 50%, Sigma-Aldrich). Hybrid cells were grown in HAT medium (RPMI 1640 supplemented with 15% heat-inactivated FBS, 100 mM hypoxanthine, 0.4mM aminopterin, 16 mM thymidine, 1 mM Na Pyruvate, 2 mM Ultraglutamine and 1% Antibiotic/antimycotic) and supernatants were tested by ELISA and flow cytometry.

10 Hybridoma screening

ELISA screening was performed as previously described, with a slight modifications: 50 µL of whole cells supernatant were added to a single peptide-coated well. Positive control was mouse positive serum diluted 1:1000 in PBS-10% FBS, while negative controls were mouse serum at day 0 diluted 1:50 in PBS-10% FBS and PBS-10 % FBS alone.

Flow cytometry screening was performed staining $5x10^3$ GRANTA-519 cells in a p96 multiwell plate (BD Falcon, USA) with 50 of cells supernatant for 20 minutes at 4

℃. The plate was then washed with 100 µL/well of PBS, centrifuged at 1200 rpm for 2 minutes and the supernatants were discarded. Secondary FITC-conjugated antibody was added (0.5 µL/well in 50 µL/well Staining Buffer) for 20 minutes, in ice, in the dark, and, after washing, cells were resuspended in 200 µL of PBS and analyzed by flow cytometry.

11 Hybridoma growth and expansion

Since our main goal was to identify antibodies able to stain BARF1-positive tumour cells, we chose to favour flow-cytometry positivity to ELISA reactivity. Flow-cytometry-positive hybrid cells were expanded and slowly converted into HT medium (RPMI 1640 supplemented with 15% heat-inactivated FBS, 100 mM hypoxanthine, 16 mM thymidine, 1 mM Na Pyruvate, 2 mM Ultraglutamine and 1% Antibiotic/antimycotic) and then into RPMI complete medium. In some cases, we were also able to convert hybridomas into a synthetic FBS-free medium (IMDM), which resulted in a more efficient immunoglobulin purification.

12 Isotyping

The isotype characterization of the antibodies was performed using IsoStrip Mouse Monoclonal Antibody Isotyping Kit (Roche) following the manufacturer procedures: 150 µL of cells supernatant diluted 1:30 in PBS-1% FBS were added into a development tube; reaction took place in 30 seconds at RT. An isotyping strip was added into the tube, until a blue bar appeared in the positive control.

13 Ig purification

Selected hybridomas were grown in bioreactors (CellLine, Integra Biosciences), in RPMI complete or serum-free medium. Immunoglobulins were harvested twice a week and great amounts of supernatants were obtained. Purification was performed employing SAS precipitation (Satured Ammonium Sulphate, Sigma-Aldrich): briefly, 30% SAS was added drop by drop at 4 ℃ to the Ig so lution in agitation. This led to BSA and other proteins precipitation. After centrifugation at 10,000 rpm for 60 minutes, supernatant was collected and SAS solution was added again to achieve a final concentration of 50% SAS. After centrifugation at 10,000 rpm for 60 minutes, the

pellet, containing the Ig, was resuspended in about 10 ml of PBS. Subsequently, the solution was dialyzed against 4 L of PBS in a Float-A-Lyzer G2 100 KD dialysis device (SpectrumLabs).

14 Ig analysis

Antibodies were quantified perfomed using NanoDrop (Thermo Scientific) and stored into small aliquotes at -80 ℃.

The mAb productions were evaluated on a classical SDS-PAGE gel in order to assess their purity: 500 ng and 1 µg of each mAb were loaded on a pre-cast 10% Acrylamide gel (Invitrogen) in 15 µL final volume of LB Buffer (50 µM Tris-HCl, pH 6.8; 2% lauryl sulphate, 50 mM DL-Dithiothreitol, 10% glicerol, 0.1% Bromophenol blue, all from Sigma-Aldrich). We used SeeBlue Marker (Invitrogen) as a molecular marker. After run, gel was stained with LB Brilliant Blue (Sigma-Aldrich) for 30 minutes at RT, and excess staining solution was washed with Washing Buffer (45% water, 45% Methanol, 10% Glacial Acetic Acid, all from Carlo Erba) several times for 90 minutes. Gel was then dried up at 80℃ for 2 hours and scann ed.

15 Ig *in vitro* characterization

15.1 Flow cytometry

We used both EBV-positive and -negative cell lines: GRANTA-519, C-666, B95.8 and BL-41 B95.8 cells were used as positive controls, while BL-41 and Raji cells were the negative controls. We stained $2x10^5$ cells with approximatively 1 µg of anti-BARF1 mAb for 15 minutes on ice. Cells were then washed and labeled with a secondary FITC anti-mouse IgM or IgG (BioLegend), depending on the mAb isotype, for 15 minutes on ice. Cells were then washed and analyzed by FACSCalibur flow cytometer. Analysis was performed with FlowJo (TreeStar) software.

15.2 Complement-Dependent Cytotoxicity

Target cells ($6x10^5$ GRANTA-519, C-666, and Raji cells) were stained with 100 µCi $Na_2\,^{51}CrO_4$ (Perkin-Elmer) for 1 hour and 30 minutes at 37 ℃. Cells were then

washed twice on FBS gradient and resuspended in Staining Buffer. We seeded radiolabeled cells at $2x10^3$ cells/well in triplicates in 50 µL Staining Buffer in a 96-well plate (Sarstedt) and stained with approximatively 1 µg anti-BARF1 mAb in 50 µL Staining Buffer for 1 hour at 4 ℃. After washing, supernatant was discarded and cells were resuspended in 200 µL RPMI-25% Human Serum (non-heat inactivated, thus retaining all the complement proteins still active; Lonza), for 1 hour at 37 ℃. Negative controls (or spontaneous release) were unlabeled cells, while for positive control (maximum release) we added 100 µL Triton 5% (Sigma-Aldrich). At the end of the incubation, we collected 100 µL of supernatant and evaluated the radioactivity by using a γ–ray counter (Cobra Gamma Counting System, Packard Instrument Company). The cytotoxicity index was evaluated as follows:

$$C.I. = 100 \times \frac{\%test - \%spont}{100\% - \%spont}$$

where *%test* is the percentage of cytotoxicity obtained with mAb plus complement, while *%spont* is the percentage of cytotoxicity of complement alone.

15.3 Antibody-Dependent Cell-Mediated Cytolysis (ADCC)

ADCC was performed following Calcein-AM (Invitrogen) protocol. Briefly, $1x10^6$ target cells were resuspended in 1 ml Hank's Balanced Salt Solution added with 5% FBS (HBSS-FBS, 5.4 mM KCl, 0.3 mM Na_2HPO_4, 0.4 mM KH_2PO_4, 0.2 mM $NaHCO_3$, 0.5 mM $MgCl_2$, 0.4 mM $MgSO_4$, 137 mM NaCl, all from Sigma-Aldrich) and labeled with 7.5 µL of Calcein-AM 1 mg/ml for 30 minutes at 37 ℃. After 3 washings, cells were resuspended in 1 ml HBSS-FBS: 300 µl of cells suspension were diluted with 200 µl of HBSS-FBS, and we added anti-BARF1 mAb at a concentration of 20 µg/ml, 10 µg/ml and 5 µg/ml, for 30 minutes in ice (negative controls were performed with HBSS-FBS only). As a positive control, target cells were lysed with Triton 5%. Finally, cell suspension was diluted to 3 ml with HBSS-FBS and cells were plated 100 µl/well on a U-bottom 96-well plate. Effector cells were freshly thawed PBMC from healthy donors, and they were seeded at different effector-target ratios (300:1, 150:1 and 75:1) for 4 hours at 37℃, then 100 µl of supernatant were collected and seeded on a 96-well Black OptiPlate (Nunc). After 15 minutes at RT, the plate was read at 485 nm (differential read at 535 nm) using Victor X Multilabel Plate reader (Perkin-Elmer).

Lysis percentage (% Lys) was calculated as follows:

$$\%Lys = 100 \times \frac{test - spont}{\max - spont},$$

where *test* is the experimental value, *spont* is the value of untreated target cells and *max* is the value of positive control.

15.4 Western Blot

Western Blot allows the identification of protein previously separated on a SDS-PAGE gel according to their molecular weight. In this study, we used Western Blot in order to evaluate whether our anti-BARF1 clones were able to recognize BARF1 monomer.

We lysed 5x10^5 GRANTA-519, BL-41 and BL-41 B95.8 cells by adding 15 µL of LB Buffer and incubating for 15 minutes at 100 °C. Cell lysates were then loaded onto a pre-cast 10% Acrylamide gel (Invitrogen). We loaded 10 µL of SeeBlue Marker as a molecular weight marker. After run, proteins were blotted on a PVDF membrane (Immobilion-P, Millipore) ON at 4 °C, using Xcell S ureLock Mini Cell (Invitrogen). We then proceeded with the saturation step by incubating the membrane for 1 hour in PBS - Milk 3% (Sigma-Aldrich). After washing, the membrane was incubated with anti-BARF1 mAb (diluted 1:500, 1:1000 and 1:10000 in PBS – BSA 3%). After incubation, the membrane was washed and incubated with secondary antibody HRP-conjugated goat anti-mouse Ab (1:10000 in PBS – Milk 3%, EXALPHA). After incubation at room temperature and washings, we added the substrate for HRP (SuperSignal West Pico Chemiluminescent, Thermo-Scientific) for 5 minutes, then the chemioluminescence was evaluated using ChemiDoc XRS instrument and QuantityOne (vers. 4.6) software (both from BioRad).

16 *In vivo* experiments

In order to evaluate the therapeutic activity of anti-BARF1 mAb we used tumour-cell engrafted SCID or RAG$^{-/-}$ γ-chain$^{-/-}$ mice. SCID mice present an homozygous recessive mutation of the *scid* gene, which is located close to the centromere of

chromosome 16. The mutation prevents the correct rearrangment of TCR genes (T cell receptor) in T lymphocytes and of Ig genes in B lymphocytes, thus inducing a state of lymphopoenia, hypo-γ-globulinemia and high susceptibility to bacterial, viral and other pathogen infections. The immunodeficiency makes these mice tolerant toward the engraftment of non-self tissues, since they can not generate a proper immunological response. Nevertheless, the mutation still allows the development of other cell components, like NK cells, macrophages and granulocytes.

RAG$^{-/-}$ γ-chain$^{-/-}$ mice present a mutation in *rag-1* gene. *rag-1* either activates or catalyzes the V(D)J recombination reaction of immunoglobulin and T cell receptor genes, so RAG mice, like SCID, do not have the proper set of B and T lymphocytes. Moreover, the absence of the cytokine common receptor γ-chain prevents the proper generation of NK and NKT cells too, thus providing a good model for evaluating the NK role in combination with anti-BARF1 mAb in the treatment of tumours.

Mice are kept in plastic cages, at costant temperature and with a balanced diet in the SPF (Specific Pathogen Free) animal facility of the Dept. of Oncology and Surgical Sciences, University of Padova. Procedures involving animals and their care were conducted according to the institutional guidelines in compliance with national laws (D.Lgs. n° 116/92) and CEASA (University of Padova Ethical Committee for Animal Experiments).

Six-week-old SCID mice and RAG$^{-/-}$ γ-chain$^{-/-}$ were injected s.c. with 5x10^6 GRANTA-519, C-666, BL-41 or BL-41 B95.8 cells. Mice were then divided into untretated and treated groups, receiving respectively 1 ml PBS or 1 ml anti-BARF1 mAb (in PBS, 1 mg/ml) in 5 i.p. injections of 0.2 ml each, one every two days. Tumour mass was evaluated every two days by measuring maximum and minimum diameter, and it was calculated applying the formula:

$$Tmass = \frac{d^2 \times D}{2},$$

where d and D are respectively minimum and maximum diameter. All the *in vivo* tumour-growth experiments were conducted according to the guidelines of the UK

Coordinating Committee Cancer Research (UKCCCR) (Cancer Metastasis 1989, "UKCCCR guidelines for the welfare of animals in experimental neoplasia")

17 *In vivo* imaging – Bioluminescence

In vivo imaging with bioluminescence exploits the catalysis of particular substrates in order to generate photons that can be detected by dedicated instruments. This kind of signal can be useful for monitoring the growth of tumour mass *in vivo* during time and it can be used to set up a more reliable model of pathogenesis *in vivo*. Moreover, it can be very useful in a pre-clinical therapeutic analysis, in order to give precise information on the efficacy of the treatment. In this study, IVIS Lumina II (Xenogen) was used: the machine consists of a highly sensitive CCD camera positioned above an imaging chamber. The animal bed is heated and can accomodate up to three mice at the same time, with a field of view of 5-12.5 cm.

Here, bioluminescence is used to monitor the growth of s.c. tumour masses when treated with anti-BARF1 antibodies and to track the fate of a intravenously injected B-cell lymphoma cell line (GRANTA-519), their effect on a mouse model, and the potential treatment of anti-BARF1 antibody injection.

17.1 Construction and generation of luciferase-encoding lentiviral particles

The commercial vector pGL4.10 (Promega) encoding firefly luciferase (luc2) codon optimised for more efficient expression in mammalian cells was used. The gene was excised by *BglII–XbaI* digestion and inserted into the transfer plasmid pHR'tripCMV-IRES-tNGFR-SIN (93) to yield pHR'tripCMV-luc2-IRES-tNGFR-SIN. The Fluc gene is driven by a cytomegalovirus (CMV) promoter and followed by the truncated nerve growth factor (tNGFR) gene, separated by an internal ribosomal entry site (IRES). Luciferase-encoding lentiviral vector particles were produced in 293T cells by transient cotransfection of the transfer (pHR'tripCMV-luc2-IRES-tNGFR-SIN), envelope (hCMV-G) and packaging plasmids (p8.74), as previously described (93). The vector stock was collected 48 and 72 h post-transfection, and concentrated by ultracentrifugation as already described (94).

17.2 Generation of luciferase-positive tumour cell lines

In order to use bioluminescence *in vivo*, grafted cells need to express the Luciferase enzyme, an oxidative enzyme that catalyzes the reaction $luciferin + O_2 \longrightarrow oxyluciferin + light$. In this study, cells were transduced with the lentiviral vector coding for luciferase (LV-LUX) described above: $5x10^5$ GRANTA-519 and C-666 cells were harvested and resuspended in 1 ml FBS complete medium with concentrated LV-LUX (3- to 5-fold). Cells were incubated ON at 37 ℃ in the presence of the virus, then the supernatant containing the virions was discarded and fresh medium was added. Seventy-two hours after infection, $2x10^5$ cells were collected, resuspended in 50 µL PBS and plated in a 96-well black plate (Nunc). Then, 50 µL of D-Luciferin (0.3 mg/ml, Caliper) was added to the cells for 5 minutes, and the plate was analyzed using IVIS Lumina II.

17.3 In vivo *bioluminescence experiments*

Lux-transfected GRANTA-519 and C-666 were injected s.c. in SCID mice ($5x10^6$/200 µL RPMI/mouse) at day 0. At day 7, injected mice were randomly splitted into two groups, one of which receiving 0.3 mg/mouse anti-BARF1 3D4 06/08 mAb weekly, for 3 weeks. Mice were anaesthetized with Zoletil-Rompun (0.4 mg/mouse, both from Bayer) and injected i.p. with 150 mg/kg D-Luciferin in PBS. Eight minutes after luciferin injection, mice were analyzed for photons emission using IVIS Lumina II. The same analysis was performed weekly.

In a different experiment, SCID mice were injected i.v. with $3x10^6$ LUX-transfected GRANTA-519 cells. Then, half of the mice were treated from day 7 and weekly thereafter for 3 weeks with 0.3 mg/mouse anti-BARF1 3D4 06/08 mAb. All the mice were analyzed weekly using IVIS Lumina II.

At the end of each acquisition, a photographic image was obtained. Bioluminescent pseudocolor images displayed in the text are shown superimposed on gray-scale photographic images of the mice, with the most intense detected luciferase signal shown as red and the weakest signal shown as blue.

18 Statistical analysis

The growth of tumours was evaluated using caliper, as described above. At the end of the study, control and treated group measures were analyzed. Mann-Whitney test was performed between control and treated group at different days using MedCalc, version 9.4.2.0. Survival diagrams and analysis of the survival data (using Kaplan-Meier test**) were performed with the same statistical software.**

Results

1 Peptide synthesis and conjugation

BARF1 is a 220-aa long transmembrane protein. The identification of the most immunogenic epitopes of BARF1 protein requires a great ability and hands-on experience. In order to get the most efficient and reliable peptides, a bioinformatic analysis of the whole protein was performed by Prof. Oriano Marin, from University of Padova. This analysis gave as a result 3 peptides (namely 05/08, 06/08 and 08/08), spanning all over the sequence from C- to N-terminal, as shown in figure 12. Moreover, since the cleavage of the extracellular domain takes place between aa 29 and 30, all the identified peptides reside along the secreted form of BARF1.

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MARFIAQLLLLASCVAAGQAVTAFLGERVTLTSYWRRVSLGPEIEVSWFKLGPGEEQVLIGRMHHDVIFIEWPF
RGFFDIHRSANTFFLVVTAANISHDGNYLCRMKLGETEVTKQEHLSVVKPLTLSVHSERSQFPDFSVLTVTCTV
NAFPHPHVQWLMPEGVEPAPTAANGGVMKEKDGSLSVAVDLSLPKPWHLPVTCVGKNDKEEAHGVYVSGYLSQ

CVGKNDKEEAHGVYVSGYLSQ peptide 05/08

CRMKLGETEVTKQEHLS peptide 06/08

ERVTLTSYWRRVSL peptide 08/08
```

Figure 12: Aminoacidic sequence of BARF1 protein. The sequences of peptides 05/08, 06/08 and 08/08 are highlighted.

In collaboration with Prof. Marin, peptides were sinthesized, analyzed and conjugated to KLH. All the peptides gave high yield and good KLH-conjugation rates (fig. 13 shows the absorbance values of different aliquots of the peptide 06/08 at the end of the KLH conjugation). At the end of the process, the final concentration of the three peptides was adjusted to 1 mg/ml in PBS.

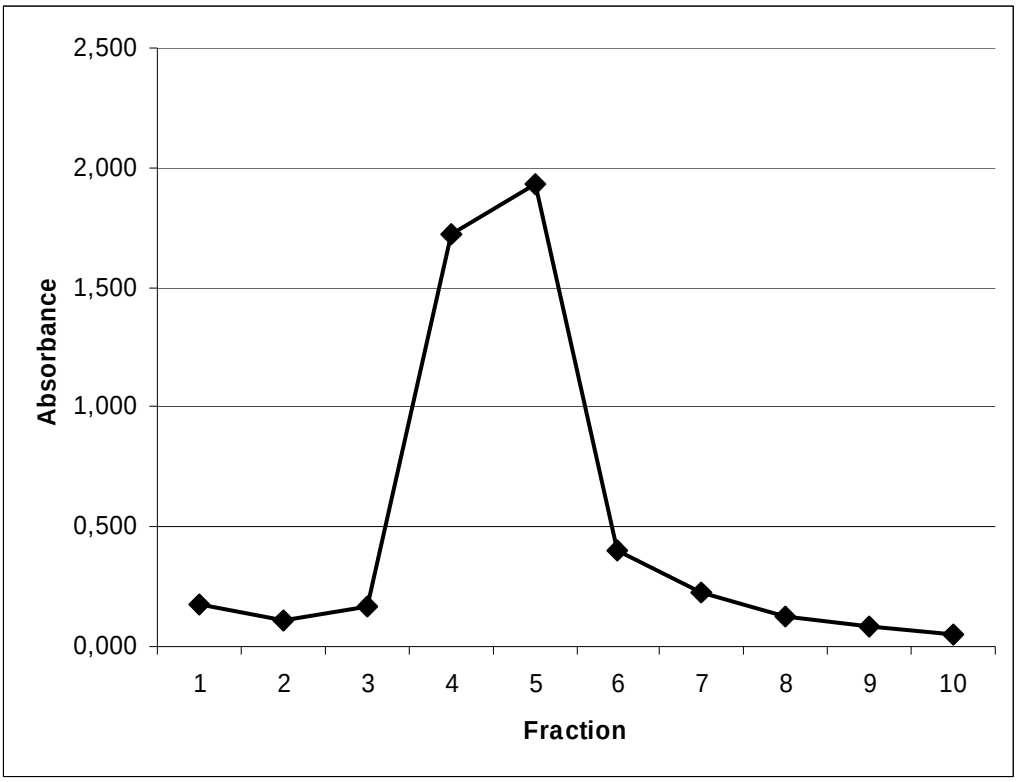

Figure 13: Graph of absorbance values of different eluted fractions after KLH conjugation to peptides and purification.

2 Peptide immunogenicity

Once we obtained the peptides, our first aim was to evaluate their immunogenicity in immunocompetent mice. Conventional BALB/c mice were immunized according to a routine schedule and sera from mice immunized with KLH-conjugated peptides (05/08, 06/08 and 08/08) were collected and analyzed by ELISA test. As reported in figure 14, which is exemplificative of 4 mice injected with KLH-conjugated peptide 08/08, all the peptides gave high absorbance values after immunisation even at very high dilution, thus demonstrating the immunogenicity of the KLH-conjugated peptides.

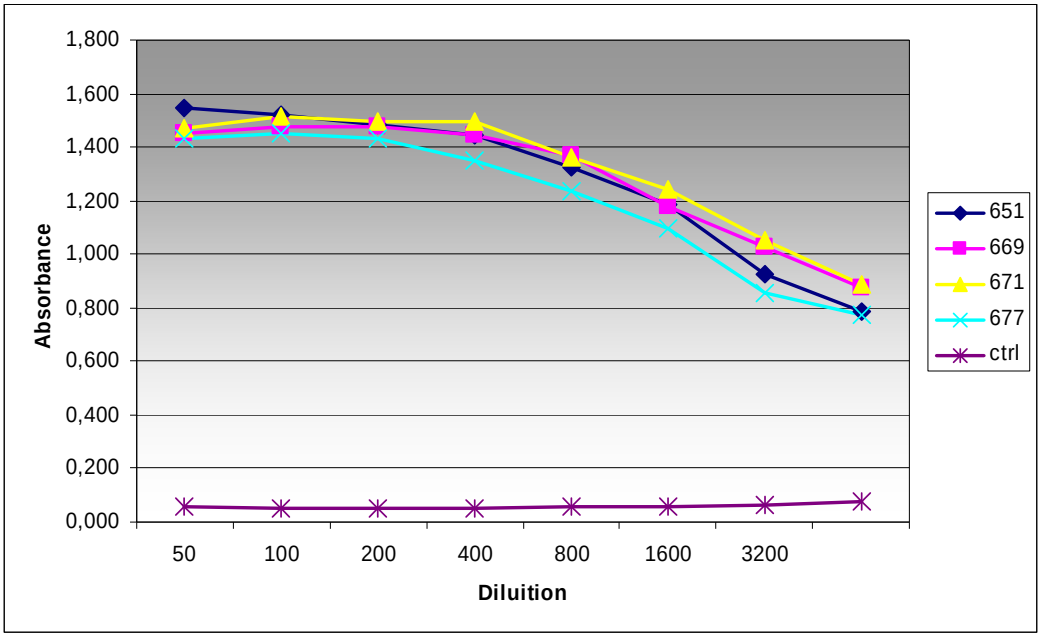

Figure 14: Graph of absorbance values of sera at different dilutions from immunized mice.

On the other side, since BARF1 is expressed on the surface of infected cells (95), we stained GRANTA-519 cell line, a human mantle lymphoma cell line expressing EBV and BARF1 mRNA (96), with mice sera and analyzed them by flow cytometry. After the first round of 3 immunisations, GRANTA-519 resulted negative, thus requiring additional immunisations of mice before a suitable signal was detected. Interestingly, immunoglobulin titres, as assessed by ELISA test, remained almost at the same levels, indicating that antibodies are already present at high titre in mice after a normal immunisation schedule, but only after repeated immunisations some Ig are able to recognize naturally folded epitopes physiologically presented on the cells surface. Figure 15 shows flow cytometry analysis of 4 mice injected with KLH-conjugated 05/08 peptide after the routine immunisation schedule and after the 3 extra immunisations.

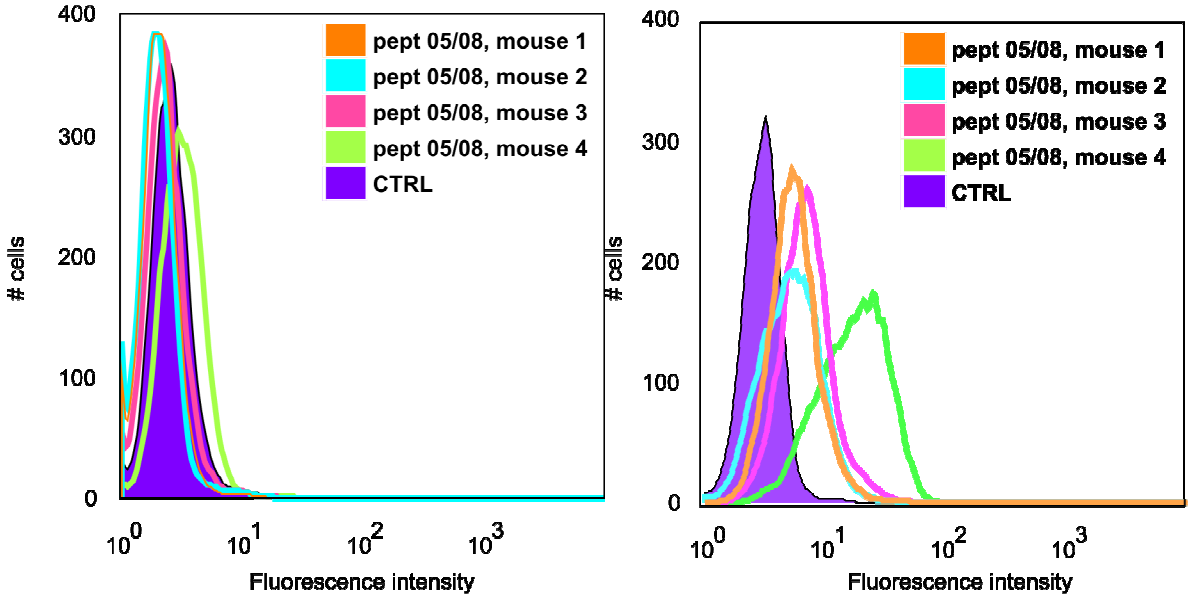

Figure 15: Left: fluorescence intensity evaluated on GRANTA-519 cells stained with sera from mice immunized 3 times with KLH-conjugated 05/08 peptide. Right: fluorescence intensity evaluated on GRANTA-519 cells stained with sera from the same mice receiving 3 additional immunisations (6 in total) with KLH-conjugated 05/08 peptide.

For every peptide, at least one mouse was selected for the hybridoma generation.

3 Hybridoma generation and screening

Mouse serum contains many different types of antibodies that are specific for many different antigens. Even in hyperimmune animals, there are seldom more than 1/10 of the circulating antibodies specific for one antigen. The use of these mixed populations of Ab creates different problems in immunological techniques. Therefore, the preparation of homogeneous antibodies with a defined specificity was a long-standing goal in immunology. This goal was achieved with the development of the technology for hybridoma production (80).

We performed several fusions of spleens from selected mice in order to obtain hybridoma clones able to target BARF1 protein. The screening was performed both by ELISA test and flow cytometry analysis, since our main goal was the identification of immunoglobulins targeting BARF1 *in vivo*. Just a few clones for every fusion were able to gave a significant signal by ELISA testing, but even fewer were able to stain GRANTA-519 cells. Notably, just one clone (clone 3D4 for peptide 06/08) was found to

be positive for both the techniques. At the end of the screening, we focused our attention only on those clones able at least to stain the GRANTA-519 cell line on flow cytometry, while those that were only positive in ELISA test were discarded.

For 05/08 peptide, only one hybridoma (10E5) was found to be positive, for 06/08 peptide we found 3 clones (3D4, 4A8 and 7E9), while for 08/08 peptide there was only one (7D9).

4 mAb selection and *in vitro* characterization

The isotyping kit revealed that all the mAb were IgM, κ chain, except for 3D4 06/08, which turned out to be IgG2a, κ chain. Since BARF1 physiologically hexamerizes (71), we hypothesized that the IgM pentavalence could better link to the multimer, thus explaining the relative abundance of the IgM isotype we obtained. On the other side, the IgG isotypes are much more common and useful both in clinical and in pre-clinical tests, and it appeared to be very promising for our studies.

Selected clones were then expanded using a commercial bioreactor, and greater amounts of antibodies were produced for the subsequent characterization. SDS-PAGE revealed that immunoglobulins were almost pure, with traces of BSA, as indicated in fig. 16, where 3D4 clone SDS-PAGE is shown as an exemplificative gel.

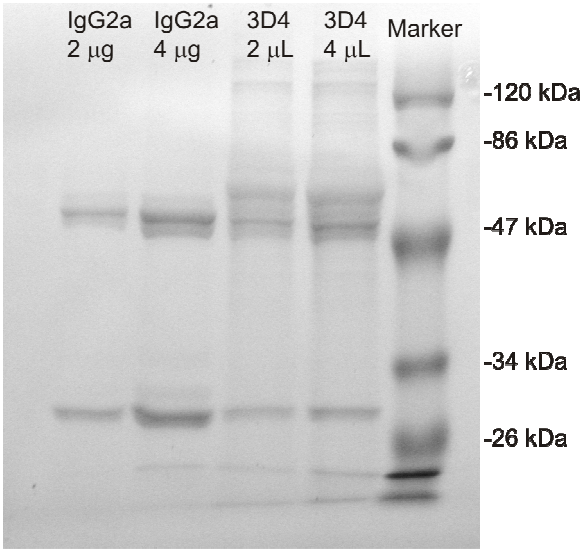

Figure 16: SDS-PAGE of different amounts of 3D4 mAb ("3D4" lanes) and of a control IgG2a commercial immunoglobulin ("IgG2a"). In all the lanes, there are two main bands, at around 30 kDa and 50 kDa, corresponding to the light and heavy chain respectively. In the "3D4" lanes , the extra bands correspond to some traces of BSA.

5 Flow cytometry analysis

After the identification of some suitable clones, they were deeply analyzed by flow cytometry for their ability to stain a panel of cells: GRANTA-519, C-666, B95.8, and BL-41 B95.8 were used as positive control cells, while RAJI and BL-41 were negative controls. In particular, RAJI is an EBV positive cell line presenting a deletion of BARF1 sequence, while BL-41 cell line is an EBV-negative Burkitt's lymphoma cell line. Although with different staining abilities, all the selected antibodies retained the ability to stain BARF1-positive cells, while BARF1-negative cells remained negative (fig. 17 and table 1).

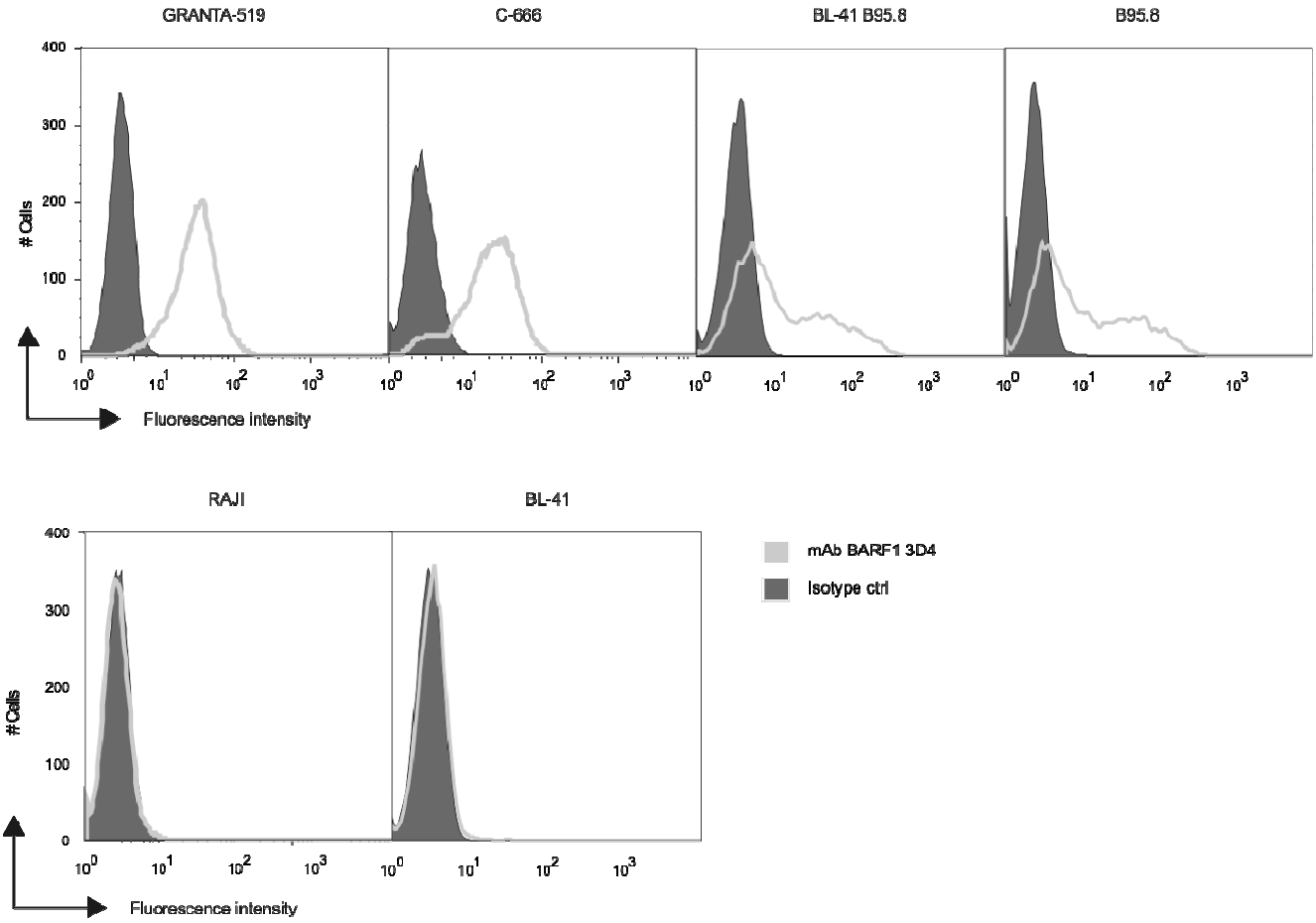

Figure 17: Flow cytometry analysis of 4 EBV-positive (C-666, GRANTA-519, BL-41 B95.8, and B95.8) and 2 -negative cell lines, stained with anti-BARF1 3D4 clone. All the EBV-positive cell lines, albeit at different percentages of positivity and MFI, resulted positive for BARF1 staining, while the EBV-negative cell lines remained negative.

Cell line	% Fluorescence	MFI	Cell line	% Fluorescence	MFI
GRANTA	99.98%	49.21	RAJI	1.28%	1.61
C-666	81.65%	29.82	BL-41	1.8%	1.1
BL-41 B95.8	52.7%	29.11			
B95.8	50.51%	23.99			

Table 1: Fluorescence percentages and MFI of 4 EBV-positive (C-666, GRANTA-519, BL-41 B95.8, and B95.8) and 2 -negative cell lines, stained with anti-BARF1 3D4 clone.

The differences in staining the positive cells can be likely ascribed to the differential expression of BARF1: indeed, few information is available about BARF1 expression on the cell surface, so that we may expect differential protein expression on different cell lines or on the same cell line but at different culture stages (in fresh medium rather then in an exhausted, acidified medium). Moreover, the cleavage of extracellular BARF1 domain has been reported, even if the cleavage rate is still to be elucidated (97). We are now setting up a new cellular model: EBV negative cell lines (like BL-41, but also 293T, which is a human embryonic kidney cell line) are going to be transduced with BARF1-coding vectors. These cells will be then evaluated for BARF1 mRNA expression and then for BARF1 protein expression using anti-BARF1 mAb.

Among all the tested clones, clone 3D4 gave the best results: the most positive stainings were obtained against GRANTA-519 (99.98% positivity) and C-666 cell line (81.65% positivity), but also BL-41 B95.8 and B95.8 gave good percentages of positivity (52.7% and 50.51% respectively). On the other hand, BARF1 negative cells were not stained by 3D4 clone, thus highlighting the specificity of this mAb.

6 Western Blot

Antibodies were also evaluated for their ability to mark BARF1 from tumour cells after SDS-PAGE and blotting on nitrocellulose membrane. To this end, we tested three concentrations of the different clones against EBV-positive and -negative cells. Noteworthy, not all the clones were able to give a positive band: in particular, only clone 10E5 gave a band (with the dilution of 1:1000, fig. 18) in EBV-positive cells lane,

even if the molecular weight is higher than expected. This may be due to the glycosylation of the extracellular domain of the protein, which is not removed after the lysis of cells. For all the other clones, we were never able to obtain a visible band at the expected molecular weight, thus meaning that the remaining clones probably recognize conformational epitopes of BARF1 and not linear ones.

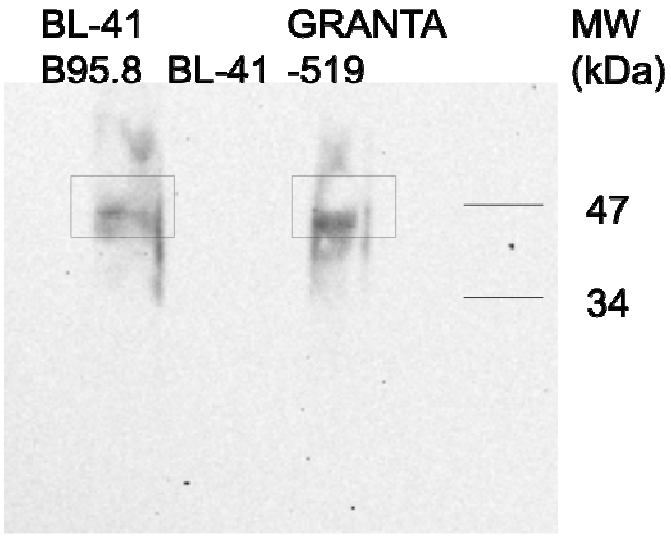

Figure 18: Western Blot of 2 EBV-positive cell lines (GRANTA-519 and BL-41 B95.8) and 1 EBV-negative cell line (BL-41) using anti-BARF1 10E5 clone. Only in the lanes corresponding to the EBV-positive cell lines it is possible to appreciate a band at around 40 kDa while the molecular weight of BARF1 is around 25 kDa. This may be due to the glycosylation of the extracellular domain of the protein.

7 Complement-dependent cytotoxicity

CDC is a modality of action of many monoclonal antibodies currently used in therapeutic clinical protocols (98). It is a mechanism of killing cells in which antibodies bound to the target cell surface fix complement, thus resulting in assembly of the membrane attack complex that punches holes in the target cell membrane with subsequent cell lysis.

Complement mediated lysis was assessed in a standard Chromium release assay. Even in this test, we used both EBV-positive and –negative cell lines as target cells. Although the clone 3D4 (IgG2a) is not able to trigger complement-dependant lysis (data not shown), IgM immunoglobulins were able to bind complement proteins

and to induce lysis of labeled cells. Figure 19 is a representative experiment of clone 10E5 (IgM), in the best experimental conditions (E:T ratio 300:1) and with the higher mAb concentration. EBV-positive cell lines (GRANTA-519, C-666 and BL-41 B95.8) were lysed when exposed to complement, while BL-41 (EBV-negative cell line) lysis was almost comparable to the background. As it was described for the cytofluorimetric analysis, we experienced different lysis percentages for the different cell lines, which, again, can be ascribed to the differential expression of BARF1 on the cell surface.

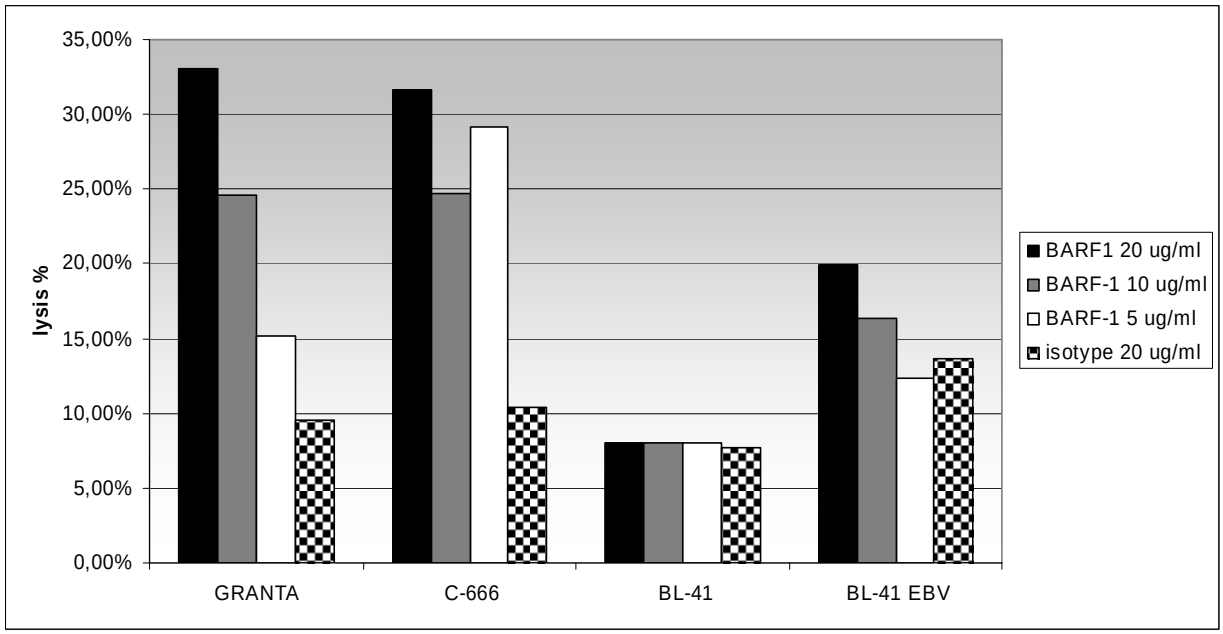

Figure 19: CDC. EBV-positive (GRANTA-519, C-666 and BL-41 B95.8) and -negative cell lines (BL-41) were treated with different amount of anti-BARF1 mAb and with the corresponding isotype. Then, HS, containing Complement proteins, was added (clone 10E5) and the lysis was evaluated in a standard ^{51}Chromium release. All the EBV-positive cell lines, albeit at different extents, were lysed, while the EBV-negative cell line was not lysed. Noteworthy, 20 μg/ml of anti-BARF1 mAb resulted in the highest lysis in all the cell lines.

8 Antibody-dependent cell-mediated cytotoxicity

This cytotoxicity mAb-mediated mechanism is typical of several antibodies already used in clinical: the mAb binds its specific target, and exposes the Fc fragment, which, in turns, binds the Fc receptors present on monocytes, macrophages, and natural killer cells. These cells then engulf the bound tumour cell and destroy it.

ADCC was performed using Calcein AM. We used the same panel of EBV-positive and -negative cells already used in CDC, while as effector cells PBMC from Buffy Coat were used.

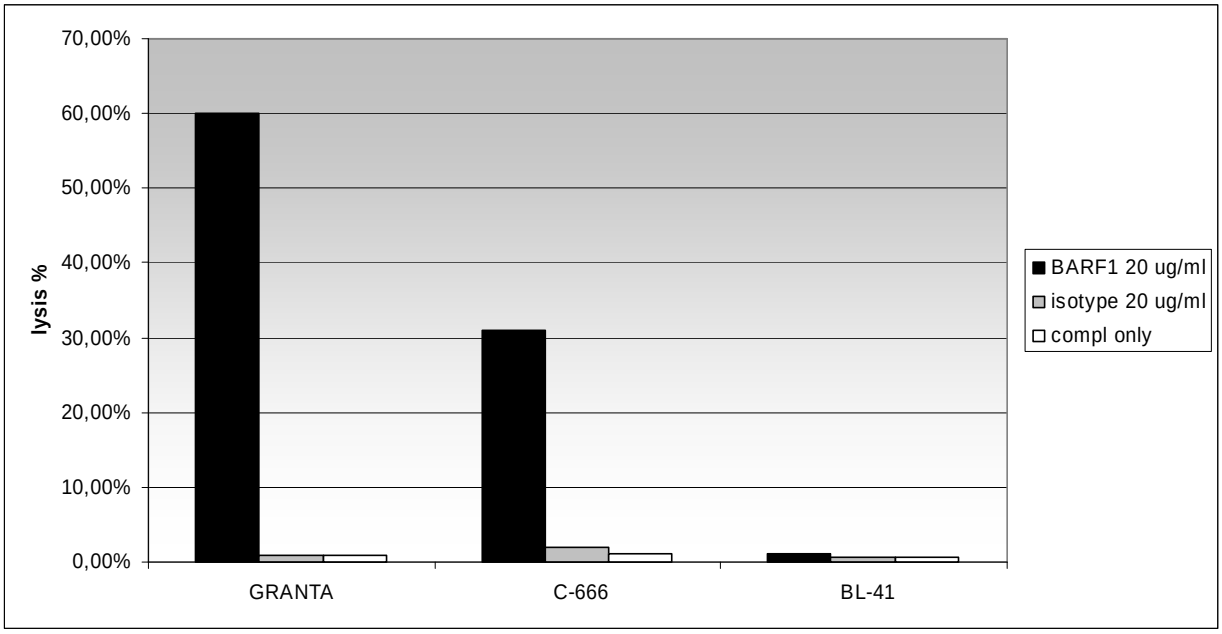

Figure 20: ADCC. Two EBV-positive (GRANTA-519 and C-666) and one EBV-negative cell line (BL-41) were stained with anti-BARF1 mAb (clone 3D4) or the corresponding isotype. Effector cells (PBMC) were added and this graph shows the results with a E:T ratio of 300:1. It is possible to appreciate that the presence of the specific antibody, but not the isotype or the absence of any antibody, mediates a marked lysis of EBV-positive cell lines, but not of the BL-41 cell line.

Figure 20 is representative of the different experiments performed. The highest BARF-1-positive target cell lysis was obtained with 20 µg/ml of 3D4 clone at an effector:target ratio of 300. In PBMC we evaluated NK population by immunophenotype (CD16 and CD56 positive): the percentage of NK cells was quantified between 12% and 15% of the total population (data not shown).

9 *In vivo* experiments

9.1 In vivo *assessment of therapeutic activity*

Since the *in vitro* tests resulted very promising, we tested the efficacy of the different anti-BARF mAb *in vivo*. SCID mice were injected with both BARF-1-positive (GRANTA, C-666 and BL-41 B95.8) and negative (RAJI and BL-41) cells and were then randomly subdivided into control and treated groups. Treatment was performed with a total of 1 mg of mAb. Prior to injection, the cell lines were evaluated by flow

cytometry to assess whether they continued to be stained by the mAb. When tumour mass became measurable, they were measured every 2 to 3 days, and mice were sacrificed when tumour mass exceeded 1500 mm^3. For every day of measurement, Mann-Whitney test was performed between the two groups.

BL-41 and BL-41 B95.8 cell lines were grafted on the opposite flanks of the same animal. Then, mice were divided into control and treated groups. The two cell lines shoved similar tumour mass growth kinetics, thus giving us the possibility to monitor both the EBV-positive and negative cell line at the same time. On the other hand, the two cell lines grew in the same way and with the same kinetics, both in the treated and in the control group (data not shown). This was probably due to the low expression of BARF1 in BL-41 B95.8 cells (see Table 1). We suppose that in this setting a greater amount of mAb woulf be required in order to appreciate some therapeutic effects.

Similarly, in mice injected s.c. with RAJI cells the treatment did not give any tumour mass reduction, as expected by *in vitro* results and literature (data not shown).

Interestingly, we obtained positive results with C-666 and GRANTA-519-engrafted mice. Five millions of C-666 cells were injected s.c. in 18 SCID mice; 9 mice were then treated at day 0 with anti-BARF1 mAb and then received a total of 5 doses of antibody in a range of 2 weeks, for a total of 1 mg of antibody. As it can be appreciated by the tumour growth kinetics, the tumour mass of treated mice grew less and slower than the control group (fig. 21). Moreover, the statistical analysis of the two groups at the different days revealed a statistically significant difference at day 24, 26 and 28 ($p=0.028$**,** $p=0.002$ and $p=0.0026$, respectively). After day 30, the therapeutic effect of the treatment dropped, and C-666 tumour mass started to grow rapidly even in the treated group. The same experiment was conducted using $RAG^{-/-}$ γ-$chain^{-/-}$ mice (these mice lack the lymphocytes components and the NK population too), but we did not observe any difference between the control and the treated group (data not shown), thus indicating that the main modality of action of the selected mAb is likely ADCC.

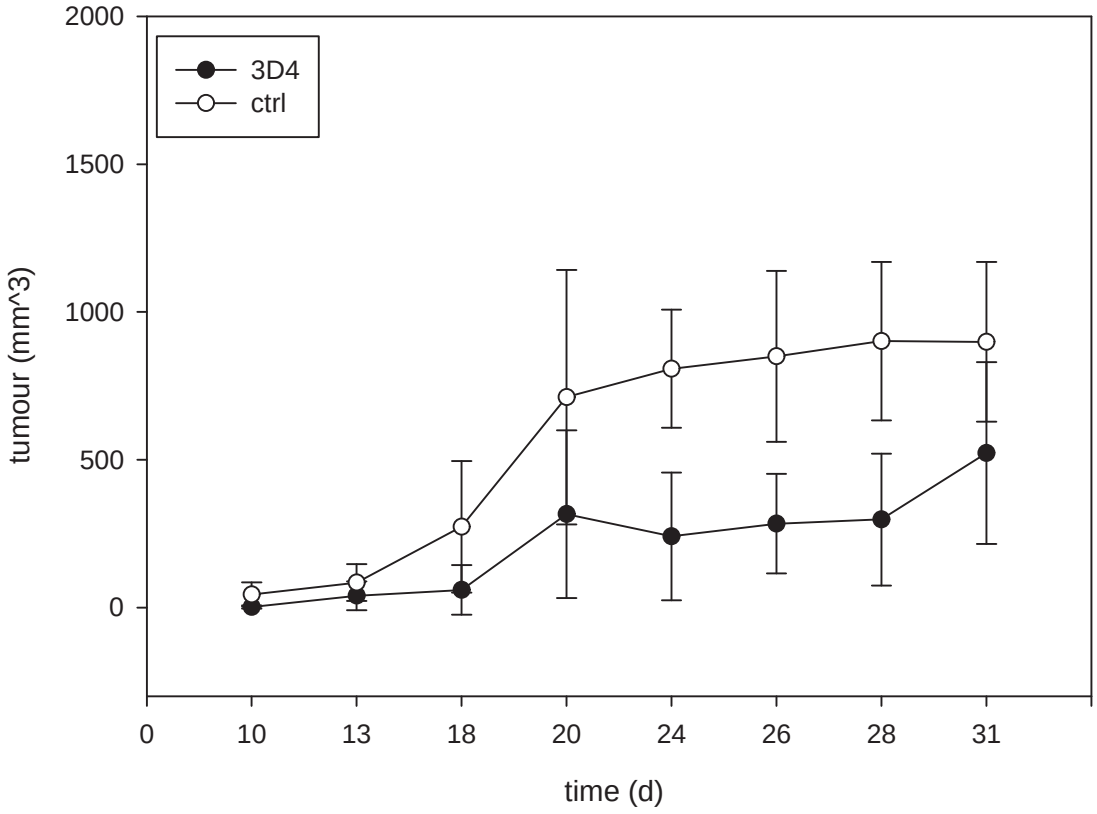

Figure 21: Growth of $5x10^6$ C-666 cells injected s.c. in SCID mice. FIve mice were untreated, while 9 mice received a total amount of 1 mg of anti-BARF1 antibody (3D4 clone). The statistical analysis performed using Wilcoxon test revealed that the reduction of the tumour growth gained by the administration of anti-BARF1 antibody is statistically significative for the day 24, 26, and 28 ($p=0.0028$, $p=0.002$ and $p=0.0026$, respectively).

In an additional experiment, $5x10^6$ GRANTA-519 cells were injected s.c. in a total of 22 SCID mice; 13 mice were then treated at day 0 with anti-BARF1 mAb and then received a total of 5 doses of antibody in a range of 2 weeks, for a total of 1 mg of antibody. As it can be appreciated by the tumour growth kinetics, the tumour mass of treated mice developed slower than the control group (fig. 22). Moreover, the statistical analysis of the two groups at the different days revealed a statistically significant difference at day 21 ($p<0.001$).

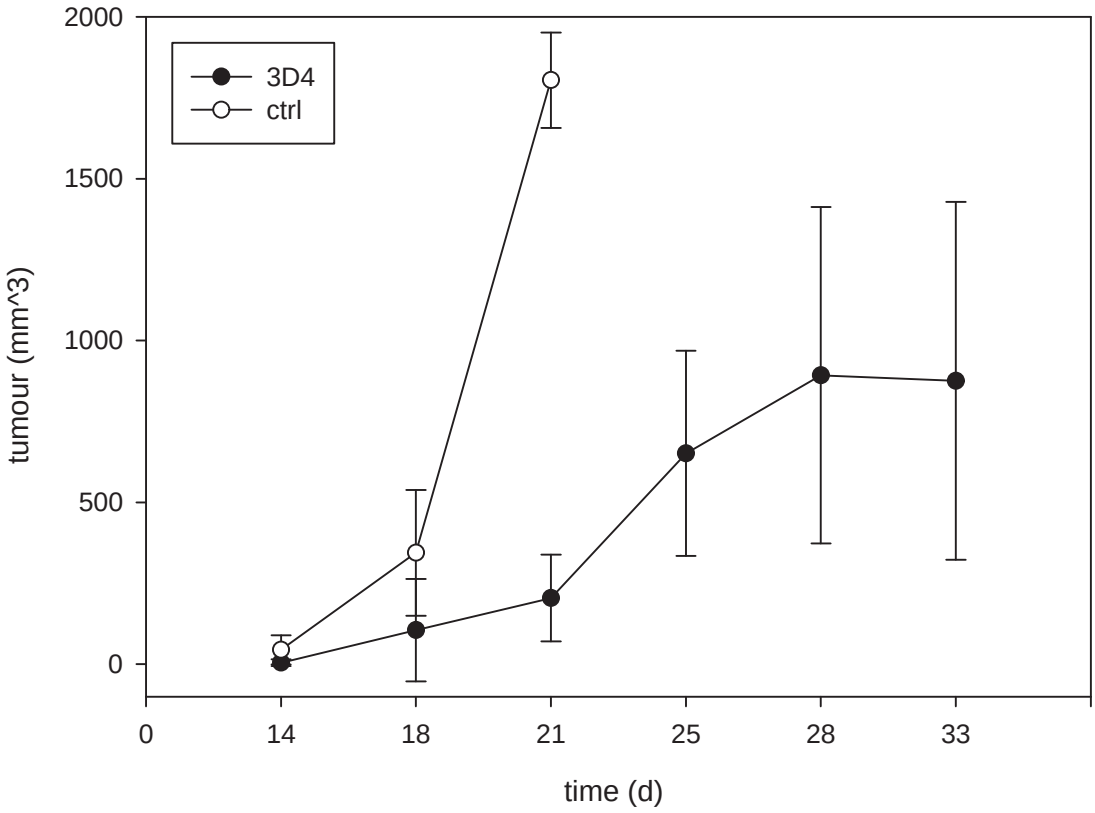

Figure 22: Growth of $5x10^6$ GRANTA-519 cells injected s.c. in SCID mice. Nine mice were untreated, while 13 mice received a total amount of 1 mg of anti-BARF1 antibody (3D4 clone). The statistical analysis performed using Wilcoxon test revealed that the reduction of the tumour growth gained by the administration of anti-BARF1 antibody is statistically significative for the day 21 ($p<0.001$).

Differently from C-666 cell line, two of the mice grafted with GRANTA-519 cell line and treated with 3D4 06/08 anti-BARF1 mAb experienced a total regression of the tumour mass and survived completely disease-free. The analysis of the survival of the two groups using Log-Rank analysis revealed a statistically significant difference ($p=0.002$), thus demonstrating the efficacy of clone 3D4 06/08 *in vivo* (fig. 23).

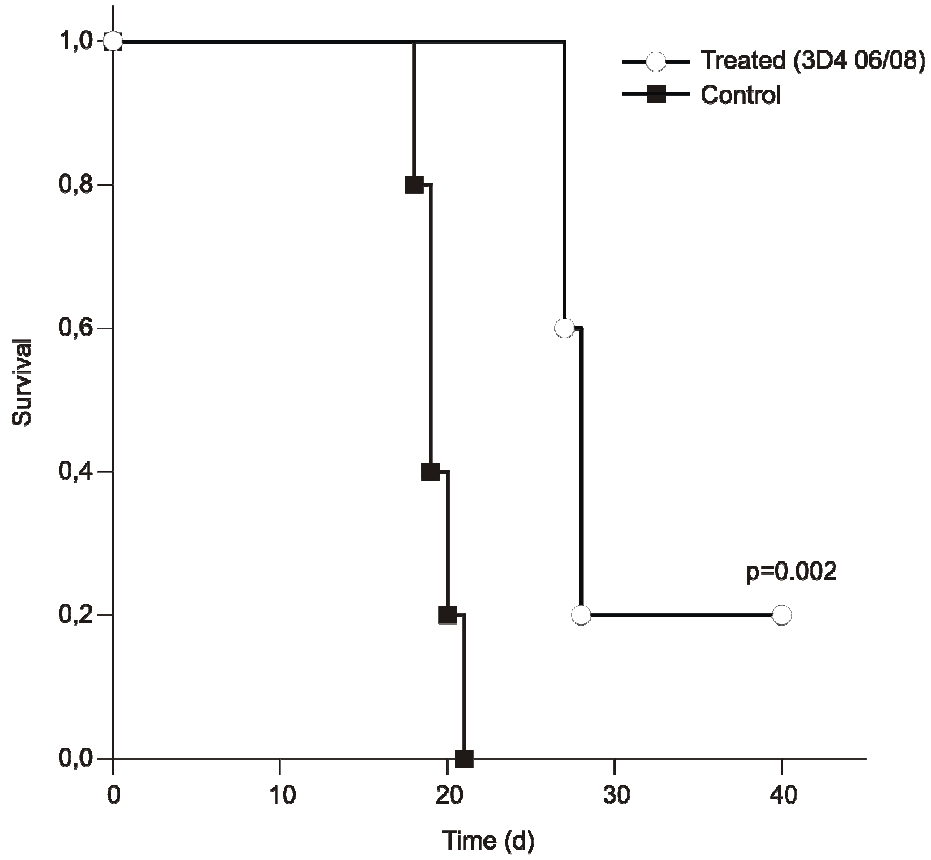

Figure 23: Survival of mice grafted with GRANTA-519 cells. The treated group received a total amount of 1 mg of anti-BARF1 mAb (3D4 clone). The Kaplan-Meier test revealed a statistically significative improvement in the survival of the treated group (p=0.002).

The same experiment conducted using RAG$^{-/-}$ γ-chain$^{-/-}$ mice did not produce any difference between the control and the treated group, thus further supporting the notion that the mAb acts primarily through an ADCC mechanism (data not shown).

9.2 In vivo *imaging - bioluminescence*

The use of *in vivo* imaging systems allows to follow the development of the tumour and its response to therapeutic treatment, thus giving critical information about the optimization of the pre-clinical model and the validation of new therapeutic approaches. In order to visualize *in vivo* the tumour mass by using IVIS Lumina II, cells need to express the luciferase enzyme, which catalyzes the reaction from luciferin to oxyluciferin, with the concomitant emission of light.

We employed a lentiviral vector coding for luciferase (kindly gifted by dr. Stefano Indraccolo): target cells (GRANTA-519 and C-666) were transduced with the viral vector and they were analyzed after 48 hours. The two cell lines gave an equal signal

when analyzed using IVIS Lumina II (about 10^5 Ph/sec for $2x10^5$ cells, in a 96-well black plate), so we decided to expand the cells and inject them in SCID mice. We used the same amount of cells ($5x10^6$) and the same delivery route (s.c.) used for the previous *in vivo* experiments. Animals were then anaesthetized and injected with 150 mg/kg D-luciferin. Eight minutes later, mice were analyzed using IVIS Lumina II weekly. We decided to modify the treatment schedule of the treated group, by giving weekly shots of about 0.3 mg of mAb starting from day 7, in order to widen the time of action of the antibody, and to focus it during the time the masses become palpable (about 10 days after injection).

Figure 24 (top) shows the final analysis at day 14 and 49 of the mice injected s.c. with C-666-LUX. At day 14, it is possible to appreciate a more intense signal in the treated group, rather than the control one, even if the statistical analysis (figure 24, bottom) did not reveal any statistically significative difference. On the contrary, at day 49, mice belonging to the control group present a high signal in the abdomen, which was statistically higher ($p=0.001$) than that of the treated group (fig. 24, top and bottom), thus demonstrating a therapeutic activity of anti-BARF1 mAb. Data for GRANTA-519-LUX s.c. are not available yet (experiments are ongoing).

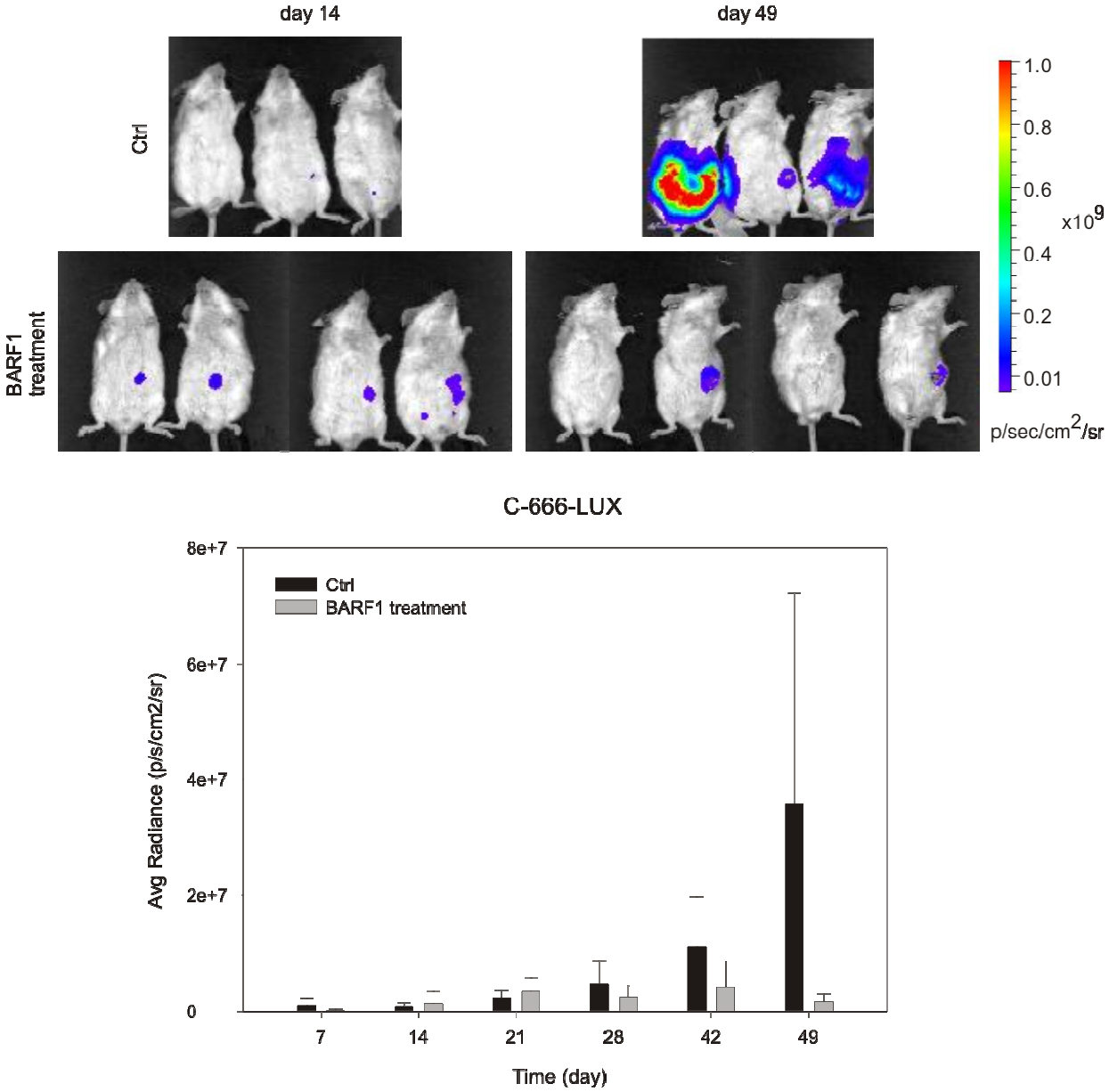

Figure 24: Top: bioluminescence analysis of mice injected s.c. at day 0 with 5x10^6 C-666-LUX cells. The images refer to day 14 and 49 of the control group (untreated) and the treated group (anti-BARF1 mAb, 3D4 clone). The values are given as radiance (p/sec/cm^2/sr). Bottom: statistical analysis of radiance from mice injected injected s.c. at day 0 with 5x10^6 C-666-LUX cells. Ctrl group was left untreated, while the treated group received anti-BARF1 mAb (3D4 clone, 1 mg). At day 49, the average radiance of the treated group is lower than the control one ($p<0.001$).

In a second set of experiments, we wanted to define a physiological mouse model for B-cell lymphoma. To this end, we injected i.v. 3x10^6 GRANTA-519-LUX in 8 female SCID mice. Animals were then anaesthetized and injected with 150 mg/kg D-Luciferin. Eight minutes later, mice were analyzed using IVIS Lumina II weekly. The

treated group received weekly, from day 7, 3 doses of about 0.3 mg/mouse of mAb 3D4 clone.

Figure 25 (top) shows the final analysis at day 14 and 21 of the mice injected i.v. with GRANTA-519-LUX. Both in the control and treated group, there are signals located at the lymph nodes, which can be regarded as a physiological homing site for B-cell lymphomas. These data confirm that the i.v. infusion of GRANTA-519-LUX cells provide a good pre-clinical model for the study of B-cell lymphomas setting.

On the other hand, the analysis performed evaluating the total number of photons in the unit of time and area, and normalized among all the scans, revealed that the total emission was significantly lower in the treated group than in the controls ($p<0.05$) at day 21, thus revealing a potential therapeutic effect of clone 3D4 in the treatment of GRANTA-519-induced B-cell lymphoma in mice (fig. 25, bottom).

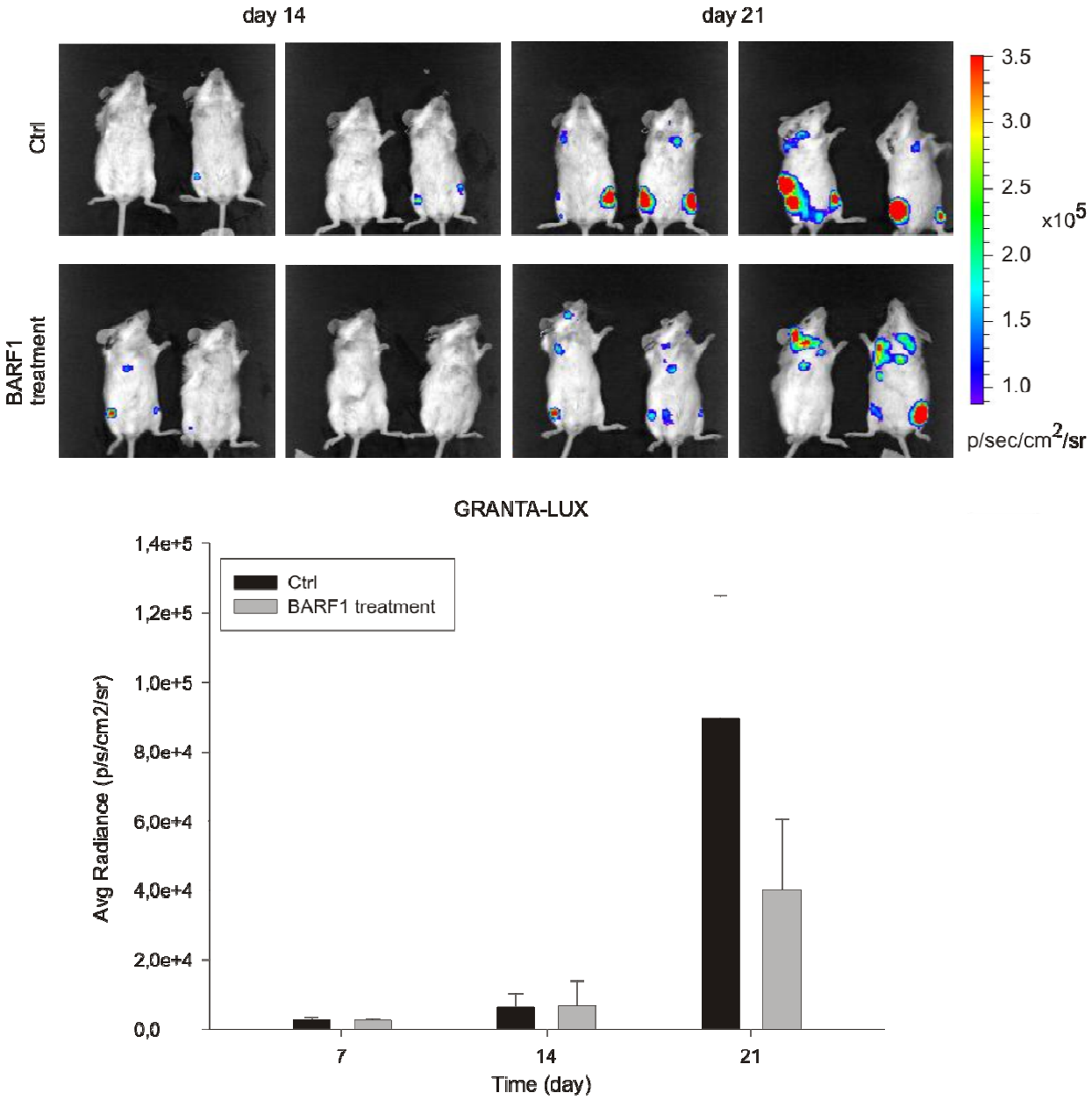

Figure 25: Top: bioluminescence analysis of mice injected i.v. at day 0 with $5x10^6$ GRANTA-519-LUX cells. The images refer to day 14 and 21 of the control group (untreated) and the treated group (anti-BARF1 mAb, 3D4 clone). It is possible to notice the presence of signals in the lymph nodes area. The values are given as radiance (p/sec/cm^2/sr). Bottom: statistical analysis of radiance from mice injected injected i.v. at day 0 with $5x10^6$ GRANTA-519-LUX cells. Ctrl group was left untreated, while the treated group received anti-BARF1 mAb (3D4 clone, 1 mg). At day 21, the average radiance of the treated group is lower than the control one ($p<0.05$).

Discussion

The idea that there might be an immune response to tumours is an old one. In the 1890's, Paul Ehrlich suggested that in humans there was a high frequency of "aberrant germs" (tumours), which if not kept under control by the immune system would overwhelm us. Later on, his theory was developed by Burnet and Thomas into the immunosurveillance concept, stating that the immune system continually surveys the body for the presence of abnormal cells, which are destroyed when recognized (99). Nevertheless, it is a common observation that even in immunocompetent patients malignancies are not naturally avoided by the action of the immune system, thus leading to the idea that the immune response may be more important toward external agents, like viruses or bacteria, rather then self malignant cells. Recently, the immunosurveillance idea was reproposed and broadened into the immunoediting theory, in which the immune system has also the active function of sculpting the immunogenic characteristics of tumors that ultimately arise in immunocompetent hosts (100). This is at the base of the immunotherapy approaches for the treatment and prevention of the neoplastic diseases.

Monoclonal antibodies (mAb) represent an attractive approach to tackling this problem as they can be designed to selectively target tumour cells and elicit a variety of responses once bound. These agents can directly kill cells by carrying toxic material to the target or can orchestrate the destruction of cells in other ways, such as activating immune system components, blocking receptors or sequestering growth factors. Therapeutic mAb have been studied in the clinic for nearly three decades. Despite inauspicious beginnings, more than 400 therapeutic mAb entered commercially-sponsored clinical development during this time, with anticancer mAb comprising approximately half of the total (101)(79). Advances in technology have vastly expanded the variety of mAb available for study, while improved understanding of cancer biology has extended the list of potential targets (102). The use of antibodies does not relies only on therapeutic strategies, but they can be used in prophilaxys settings too, and, noteworthy, for the early prognosis and staging of the disease.

The use of monoclonal antibodies in passive immunotherapeutic approaches has been hampered in the past by some technical axpects, like the production procedures, the cost of the therapy and the safety of the immunoglobulins.

For the production of monoclonal antibodies, differential methods have been developed, starting from the hybridoma technology in yest, bacterial, and mammalian cell lines. This technique is quite time-consuming and requires contaminants-free batches. Moreover, the glycosylation of the immunoglobulins may vary among the different species, so the mammalian cell cultures are preferred. Transgenic mice provide an alternative and potentially cheaper source of production, as compared to the traditional methods.

The costs for the start-up, the production of high yield, large amounts of antibodies are very high, and it is extimated around 400-500 millions of euros (103) and requires 5 to 10 years. The cost of the whole process explains the high costs of the monoclonal antibodies currently used in therapy.

Last, safety of these new therapeutics has yet to be fully elucidated, since large doses of mAb are usually employed in every treatment: besides the heavy costs, it can give problems concerning administration, bioavailability and immunogenicity.

Besides these limitations, more than 400 new therapeutic immunoglobulin entered clinical trials, which is also due to new production and screening approaches, like phage-display. The potential of the monoclonal antibodies for the treatment of malignancies resides mainly on their specificity: the cases of side effects related to the use of mAb are lower than with conventional drugs. Furthermore, chimaeric or humanized antibodies, whose chains are mutated so that they resemble human immunoglobulins, are currently in use: this approach augments mAb half-life and reduces the likelihood of HAMA, human antibodies anti-mouse antibodies, responses.

For the treatment of EBV-related malignancies, the mAb currently most used in clinics is rituximab (anti-CD20 chimaeric antibody). Its main action is directed towards CD20 expressing cells, namely the B lymphocytes. Rituximab proved to be a very potent therapeutic tool for the treatment of EBV-positive or -negative B-cell lymphomas (67, 104), even if its target is not specific of malignant cell, but it is shared among all the B cells. This lead in same cases to the development of cytopenias and hypogammaglobulinemia (105). The need of a new and more specific tool is required for the treatment of this class of malignancies. In particular, the identification of an

antigen specific for EBV-infected cells might be useful for the treatment of the diseases related to this virus. To this end, a very promising target appears to be BARF1, a viral protein expressed on the surface of infected cells. While the protein belongs to the lytic phase antigens, nevertheless it has been recently associated to some types of EBV-related carcinomas, like nasopharyngeal and gastric carcinoma.

In this work, we describe the generation of new mouse monoclonal antibodies raised against different epitopes of BARF1 protein. The immunoglobulins proved to be efficient when tested *in vitro* for the staining of EBV-positive and –negative cell lines. Moreover, we could demonstrate a CDC activity for the IgM isotypes and an ADCC activity for the IgG2a isotype. These preliminary data prompted us to test the efficacy of the mAb *in vivo*. The experiments were set both in SCID and $RAG^{-/-}$ γ-$chain^{-/-}$ mice, previously grafted with the tumour cell lines. Even if at different extents, tumour-bearing mice treated with anti-BARF1 monoclonal antibodies underwent a reduced tumour mass growth, when compared to the control groups. Moreover, the use of clone 3D4 06/08 induced the complete regression of the mass in 2 of the treated mice. These data are very promising for the use of this particular clone for further experiments.

In order to evaluate a different pre-clinical model, we also transduced GRANTA-519 cells (human B-cell lymphoma cell line, EBV-positive) with the luciferase gene. GRANTA-519-LUX were injected i.v. in SCID mice and analyzed weekly using IVIS Lumina II. Half of the mice were also treated weekly with anti-BARF1 3D4 06/08 clone. Bioluminescence analysis revealed that the total emission of the control group was significantly higher than the treated group, which means that in the anti-BARF1 treated group the tumour cells grew less than in the control group. This result is very promising, and we believe that the use of higher doses of antibody may be useful for the treatment of tumour-bearing mice.

In the future, thanks to the collaboration with Dr. Riccardo Dolcetti's laboratory at CRO – National Cancer Institute, we plan to transduce EBV-negative cell lines with the BARF1 gene: transduced and untransduced cells can be better exploited for the assessment of the specificity of the mAb. In fact, the staining of BARF1-transduced cells with our mAb will be the final proof of their specificity.

Moreover, we plan to procede with some modifications of the binding site and of the backbone of the mAb. The mutagenesis of single residues of the idiotype will allow to identify those clones presenting a higher affinity for their substrate: affinity may not

be important only from a mechanistic perspective, e.g. to allow an antibody that blocks a ligand–receptor interaction compete more efficiently, but an increased affinity may also lead to a reduced dosage of a therapeutic mAb, a cost-effective argument obviously important for patients and Health Services. On the other side, the use of mouse monoclonal antibodies is no longer an option in the clinical settings, because of the limited half-life of these molecules in patients, and because of the risk of the induction of HAMA. So far, the modifications of the backbone can be generated using molecular biology techniques and by inducing the expression of the immunoglobulins into mammalian cell lines. Chimaeric antibodies combine the V region of a mouse antibody with a human antibody C region, thus retaining the binding specificity of the murine antibody while presenting less foreign amino acid sequence to the human immune system. However, because the entire V domain of a chimeric antibody, about one third of the molecule, is of mouse origin, chimeric antibodies may still provoke a substantial HAMA response when used to treat humans. To reduce further the immunogenicity of murine antibodies, Winter and colleagues (106) constructed reshaped or "humanized" antibodies by combining only the smallest required part of a mouse antibody, the CDR, with human V region frameworks and C regions. A disadvantage of this approach is that the CDR may adopt a new conformation after being grafted onto the human framework so the humanized antibody often has substantially reduced affinity for the Ag. To solve this issue, the use of a screening based on the phage-display approach may help individualizing the best CDR sequence.

The importance of these new anti-BARF1 antibodies is not limited only to the therapeutic field, but it can be useful for the early detection of NPC and GC and could help the pathologist for the correct staging of the diseases. Moreover, we are currently broadening the use of this new tool to other EBV-related malignancies, since preclinical data indicate that also lymphoblastoid B cells (LCL, the *in vitro* model of PTLD) are positively stained by anti-BARF1 3D4 06/08 clone.

Taken together, these data indicate that BARF1 is a novel EBV-specific antigen, and that the use of anti-BARF1 monoclonal antibodies can be a potent tool for the detection and treatment of the malignancies related to the virus.

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