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BARF1 as a new therapeutic target for EBV-associated malignancies

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BARF1 AS A NEW THERAPEUTIC TARGET FOR EBV-ASSOCIATED MALIGNANCIES

ABSTRACT

While Epstein-Barr virus-specific cytotoxic T lymphocytes (EBV-CTLs) have been used successfully for the prophylaxis and treatment of the highly immunogenic post-transplant lymphoproliferative disorders, the clinical experience for other EBV-associated malignancies, such as Hodgkin's lymphoma and undifferentiated nasopharyngeal carcinoma (NPC), is limited and the results obtained so far indicate that EBV-CTLs are less effective in these settings. Decreased CTL efficacy most likely reflects immune evasion strategies by tumor cells, including down-regulation of immunodominant EBV proteins and the weak immunogenicity of the viral proteins expressed. One of the possible approaches to overcome these limitations is the identification of additional immunogenic viral proteins expressed by tumor cells that may serve as tumor-associated antigens to be targeted by improved CTL induction and expansion protocols. We have recently demonstrated that NPC patients show strong spontaneous CD4+ and CD8+ T cell responses specific for the EBV-encoded oncogenic protein BARF1. We also showed that BARF1 provides immunogenic HLA-A*0201-restricted epitopes, suggesting that exploitation of the immunogenic features of this viral antigen may help improve the current immunotherapeutic strategies for EBV-associated malignancies. On these grounds, we characterized more extensively the immunogenic properties of BARF1 with the final goal to develop improved protocols of adoptive immunotherapy based on the use of EBV-CTLs enriched in BARF1-specific effectors. In particular, we identified and validated additional BARF1 CTL epitopes presented in the context of common HLA class I alleles. These results strictly correlate to those deriving from a high-resolution HLA genotyping of a large series of NPC, giving a precise estimate of the immunogenicity of BARF1 in relation to the HLA class I profile of Italian NPC patients. To fully exploit the immunologic properties of BARF1, we are also developing and

characterizing BARF1-specific monoclonal antibodies that may be of both diagnostic and therapeutic usefulness in these clinical settings. In future perspective, the proposed research may provide a strong rationale for the clinical application of improved adoptive immunotherapy protocols for the treatment of EBV-associated malignancies, particularly the less immunogenic forms, such as NPC and, possibly, Hodgkin's lymphoma.

INTRODUCTION

EBV belongs to the human γ-herpesvirus subfamily. On infection of its target cells, EBV can undergo lytic replication during which virus progeny are released; or instead it may initiate active latency, a restricted gene expression program limited to certain members of the Epstein–Barr nuclear antigen (EBNA) and latent membrane protein (LMP) gene families and to the two Epstein–Barr small non-coding RNAs (EBER1 and 2).

Four latency types have been described depending on which of these latent genes are expressed, as shown in Table 1. Long-lived EBV-carrying memory B lymphocytes may express a putative Latency 0, characterized by complete silencing of the viral genome, or a Latency I, in which LMP-2A alone or together with EBNA-1 may be expressed (Chen F, 1995). The expression of these viral proteins is crucial for establishing and maintaining EBV persistence in B lymphocytes. An intermediate form of latency (Latency II) has been identified in B-lymphocytes that home to the germinal centers of lymphoid follicles (Babcock GJ, 2000). In these cells, the expression of EBV proteins is restricted to EBNA-1 and the three LMPs, a ‘‘rescue’’ program that provides signals allowing infected lymphoblasts to survive and differentiate into memory B cells. In the absence of effective immune surveillance, as observed *in vitro* or *in vivo* in immune suppressed patients, EBV- infected B lymphocytes show a different latency program, called Latency III, characterized by the expression of six EBV nuclear antigens (EBNA-1-6) and three latent membrane proteins (LMP-1, -2A, and -2B)(Dolcetti R, 2003). Primary EBV infection mainly takes place in the oropharyngeal region to which the virus is conveyed by saliva droplets from infected individuals (Fig 1).

	Malignancies		Mononucleosis	In vitro
type 0	EBER 1 & 2		yes	no
type I	EBNA 1	Burkitt lymphoma (Ig-myc)	yes	no
type IIa	EBNA 1, LMP 1	Hodgkin lymphoma NK/T-cell lymphoma	yes	no
IIb	EBNA 1-6	CLL in vitro infection	yes	no
type III	EBNA 1-6 LMP 1	immunoblastic post-transplant AIDS-lymphomas	yes	yes

Table 1: Latency of EBV-positive lymphomas developing in immunocompetent hosts. *Variation in the expression of EBV latent genes. Cells with restricted EBV gene expression do not have autonomous growth potential in vitro. Malignant transformation of such cells requires additional factors (cytogenetic changes, cytokines or cellular interactions). Cells with all four EBV expression programs could be detected in the lymphoid tissues of patients during the acute phase of infectious mononucleosis.*

The nature of the target cells in the oral mucosa is still controversial, but there is agreement that B cells are infected at some stage of the process. If infection is delayed to adolescence or adulthood, it can cause an infectious mononucleosis syndrome (IM), a self-resolving lymphoid disorder that is thought to result from an uncontrolled T cell reaction directed against EBV infected cells. In IM patients, EBV is exclusively found in B blasts that undergo continuous proliferation under the influence of all latent genes (latency III). Following resolution of primary infection, EBV establishes a lifelong persistence in memory B cells where the virus usually remains clinically silent. In this B cell reservoir, viral expression is entirely repressed, a process described as a passive latency or no latency. The pleiotropic nature of EBV target cells in vivo, including B and T lymphocytes, NK cells, squamous and glandular epithelia and smooth muscle cells, demonstrates that the virus possesses a very large infectious spectrum. However, and with the notable exception of B cells, primary cells and cells lines derived from these cell lineages were thought for long time to be paradoxically resistant to virus infection in vitro (Rickinson AB, 2007).

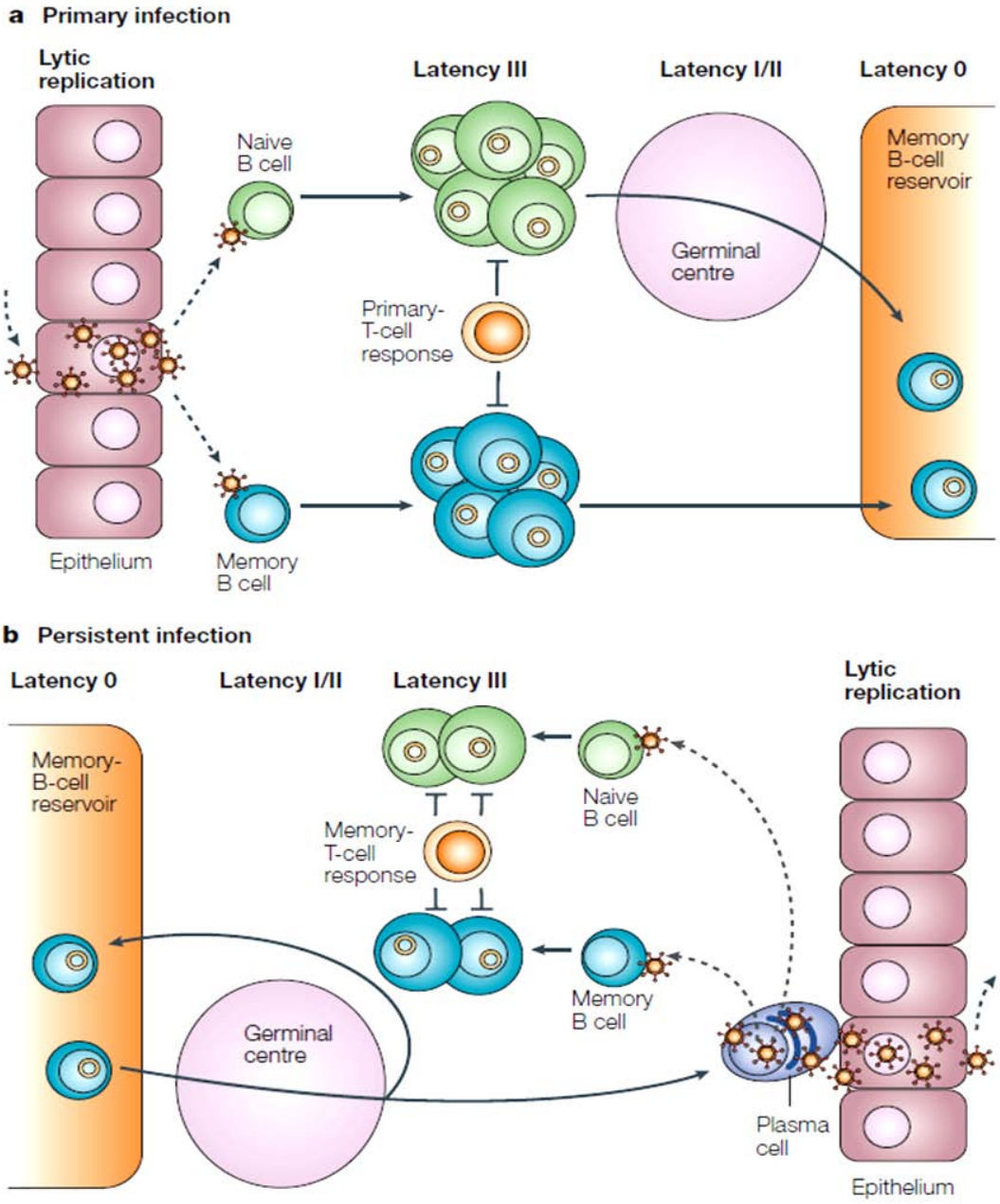

Figure 1: Putative in vivo interactions between Epstein–Barr virus and host cells.
a) Primary infection. *Incoming virus establishes a primary focus of lytic replication in the oropharynx (possibly in the mucosal epithelium), after which the virus spreads throughout the lymphoid tissues as a latent (latency III) growth-transforming infection of B cells. Many of these proliferating cells are removed by the emerging latent-antigen-specific primary-T-cell response, but some escape by downregulating antigen expression and establishing a stable reservoir of resting viral-genome positive memory B cells, in which viral antigen expression is mostly suppressed (latency 0).* ***b) Persistent infection.*** *The reservoir of EBV-infected memory B cells becomes subject to the physiological controls governing memory-B-cell migration and differentiation as a whole. Occasionally, these EBV-infected cells might be recruited into germinal-centre reactions, entailing the activation of different latency programs, after which they might either re-enter the reservoir as memory cells or commit to plasma-cell differentiation possibly moving to mucosal sites in the oropharynx and, in the process, activating the viral lytic cycle. Virions produced at these sites might initiate foci of lytic replication in permissive epithelial cells, allowing low-level shedding of infectious virus in the oropharynx, and might also initiate new growth transforming latency III infections of naive and/or memory B cells; these new infections might possibly replenish the B-cell reservoir, but are more likely to be efficiently removed by the now well established memory-T-cell response.*

EBV-ASSOCIATED DISEASES

INFECTIOUS MONONUCLEOSIS

EBV is one of a variety of infectious agents that can induce a mononucleosis syndrome. IM may be misdiagnosed as a high-grade large B cell lymphoma, or even Hodgkin's lymphoma, particularly in cases containing a large number of B immunoblasts, some of which express CD30, and rare Reed–Sternberg like cells (Gatter K, 2002). EBVinfected B cells in IM show a typical latency III expression pattern including EBER, EBNA1, EBNA2 and LMP1, which renders virus detection very easy. The mere presence of EBV does not however exclude a large B cell lymphoma, but detection of EBNA2 expression in infected cells strongly suggests the diagnosis of IM. In these cases, analysis of EBV serology establishes the proper diagnosis.

EBV-ASSOCIATED TUMORS IN IMMUNOSUPPRESSED INDIVIDUALS

The role of the immune system in controlling EBV latent infection is best illustrated by the observation that individuals with a congenital or acquired (eg: after HIV infection or organ transplantation) immune deficiency are at increased risk for the development of EBV-associated diseases, whose histological type varies according to the type and the severity of immunodeficiency. In these cases, the clinical context is more suggestive for the diagnosis of this category of tumors than the detection of EBV alone (Carbone A, 2005). However, assessing viral status can have important consequences for treatment of the disease, as EBV positive-lesions are in principle amenable to EBV-specific T-cell therapy or even to anti-viral therapy.

Post-transplant lymphoproliferative disorders. Post-transplant lymphoproliferative disorder (PTLD) is thought to result from iatrogenic immune suppression and chronic antigenic stimulation from the engrafted organ after hematopoietic stem cell or solid organ transplantation (Fig 2). PTLD includes a wide spectrum of diseases ranging from polyclonal reactive lymphoid hyperplasia to monoclonal malignant lymphoma. About 80% of PTLDs are associated with EBV. The overall incidence of PTLD is about 1% in patients with hematopoietic cell transplantation and less than 2% in those with solid-organ transplantation. The incidence of PTLD after solid-organ transplant varies depending on the age of the

recipient and the types of allograft: in adults, PTLD is more common after lung and small bowel transplantation (Pickhardt PJ, 1999). PTLD is more common in pediatric patients, largely because children are more likely to be primarily infected with EBV via the graft and the risk of developing PTLD is greatest during the first year after transplantation and declines thereafter. PTLD after hematopoietic cell transplantation presents as a widespread disease involving nodal and extra-nodal sites, while PTLD after solid-organ transplantation frequently involves the allograft itself, which suggests a lymphomagenetic role of chronic antigen stimulation occurring within the graft (Tao Q, 2006). Other sites are also frequently involved, such as the gastrointestinal tract, liver, lungs, lymph nodes, bone marrow, skin, and CNS. Diagnosis of PTLD should be based on careful pathological examination, preferably on excision biopsy specimens (Pickhardt PJ, 1999). Reducing immune suppression to restore EBV-specific immunity, with close monitoring for acute rejection, is the key to successful management of PTLD. Chemotherapy and anti-B-cell antibodies may be used in combination with the reduction in immune suppression, whereas surgery may be considered for localized PTLD. Reduction of immune suppression alone results in clinical remission in 25–63% of adult and in 40–86% of pediatric PTLD patients. The prognosis of PTLD not responding adequately to the reduction in immune suppression is poor, with mortality exceeding 50% (Pickhardt PJ, 1999).

HIV-associate lymphomas. Patients with HIV infection are at 60- to 200-fold increased risk of developing various lymphomas. Lymphoma is the first acquired immunodeficiency syndrome (AIDS)-defining illness in 3–5% of HIV+ patients (Carbone A, 2005). EBV positivity is significantly correlated with HIV disease status, subtypes of HIV-associated lymphoma. HIV-related lymphomas tend to involve extra nodal tissues, in particular the gastrointestinal tract, lungs, liver, CNS, and bone marrow. The overall incidence of EBV-positive cells in HIV-related lymphomas is about 60%, but the incidence varies with the site and the subtype of lymphoma.

- *Primary effusion lymphoma (PEL)* is a peculiar type of lymphoma affecting immunocompromised patients, especially those with HIV infection (Taylor AL, 2005). PEL is always associated with human herpesvirus (HHV-8)/Kaposi's sarcoma-associated herpesvirus (KSHV) and is commonly co-infected with EBV. The prognosis of PEL is extremely poor.

- *Plasmablastic lymphoma (PBL)* of the oral cavity is another type of lymphoma occurring almost exclusively in HIV patients. It is a rapidly growing lymphoma localized in the oral cavity or the jaw. EBV is found in more than 50% of cases, but no association with HHV-8/KSHV has been reported (Carbone A, 2005).

- *Hairy leukoplakia.* This benign lesion of the tongue is typically encountered in HIV-infected individuals and is clinically and histologically characterized by hyperplasia and hyperkeratosis of the tongue squamous epithelium. EBV infection is located in the upper layers of the affected epithelium where an uncontrolled, active lytic EBV replication occurs. Viral tropism for differentiated epithelial squamous cells and spontaneous EBV replication in infected cells reflect intrinsic properties of virus–cell interactions, as recently described in an in vitro model using primary epithelial cultures (Feederle R, 2007).

- *Smooth muscle tumors.* EBV-positive smooth muscle tumor (SMT) is a rare lesion and was initially described in an organ transplant recipient (Pritzker KP, 1970). SMT have so far been exclusively reported in patients with congenital or acquired immune deficiencies. Consistent EBV infection in SMT developed in organ transplant recipients and HIV-infected children was demonstrated two decades later. SMT are unusual in that they show isolated expression of EBNA2 (Lee ES, 1995). SMT are well-differentiated smooth cell tumors that can be observed in a large number of anatomical sites, but lung, liver, soft tissues, and gastrointestinal tract seem to be privileged sites. Some SMT show prominent intra-tumoral T lymphocytes which might be directed against EBV proteins. Although SMT frequently present as multiple lesions, their clinical prognosis is much more favorable than for classical leiomyosarcomas. Assessment of EBV status allows easy distinction between these two entities.

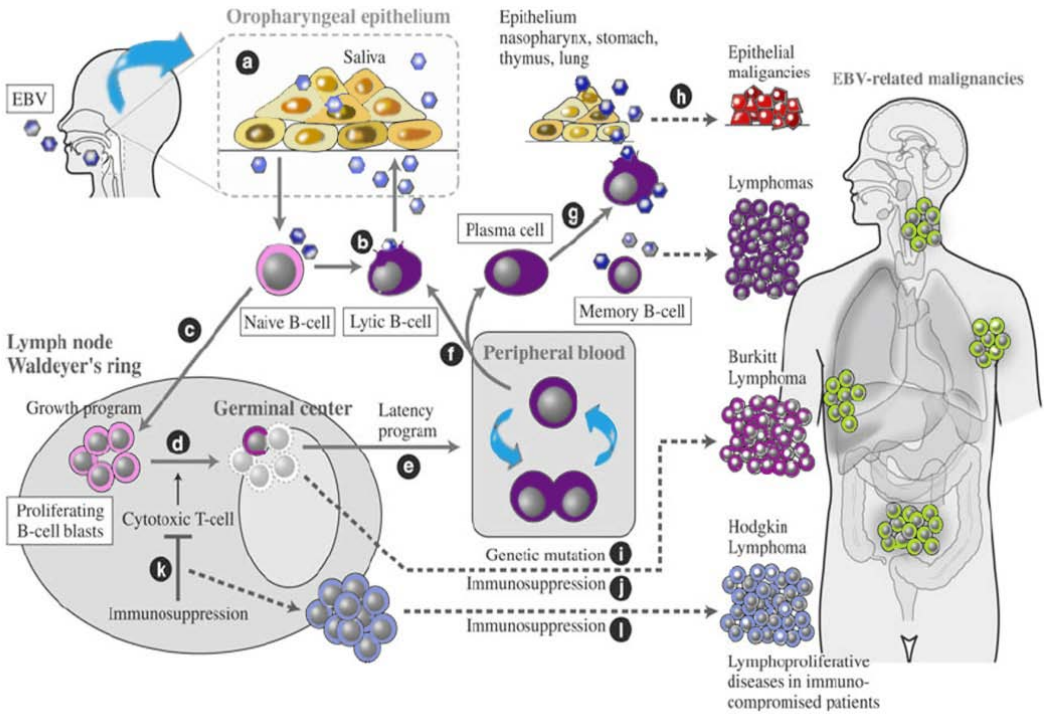

Figure 2. Epstein-Barr virus (EBV) infection and pathogenesis of EBV-related diseases. *The virus primarily infects oropharyngeal epithelium via saliva or naive B cells infiltrating mucosa (**a**). In the lytic cycle, viral particles are reproduced and shed into saliva, again infecting other mucosal 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 proliferated lymphocytes is eliminated by cytotoxic T cells before and through a germinal center reaction (**d**). Thereafter, the infected B cells express the latency program 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 (**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**). EBVs 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-center 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 center as a result of cellular mutation (**j**). In the immunosuppressive state, lymphocytes that should be destroyed in the germinal center are rescued in the absence of cytotoxic T cells (**k**). This circumstance is thought to give rise to lymphoproliferative diseases in immunocompromised patients (**l**)*

EBV AND HEMATOLOGICAL DISEASES

EBV virus has been detected in a large number of hematopoietic disorders (Fig 2).

B cell lymphomas. Systematic testing of a large panel of B cell lymphomas has shown that the EBV genome is found mainly in high-grade lymphomas, though rare cases of partial EBV infection of low-grade B cell lymphomas have been observed (Hummel M, 1995).

- *Burkitt lymphoma (BL)* was first described in children from equatorial Africa by Denis Burkitt in 1958. It is a highly proliferative B-cell tumor that includes 3 variants: *endemic* (affecting children in equatorial Africa and New Guinea), *sporadic* (children and young adults throughout the world), and *immunodeficiency related* (primarily in association with HIV infection) (Griffin BE, 2005). EBV has been detected in virtually all cases of the endemic variant, in 15-20% of the sporadic variant, and in 30-40% of the immunodeficiency-related variant (Young LS, 2004). In all variants, irrespective of the EBV status, constitutive activation of the c-myc oncogene through its translocation into one of the immunoglobulin loci is the key factor in the oncogenesis of Burkitt lymphoma (Griffin BE, 2005; Young LS, 2004). The detection of somatic hypermutations in the V region of the immunoglobulin genes and the phenotype of Burkitt lymphoma cells indicate a germinal center cell origin of this lymphoma (Küppers R, 2003). Most EBV-positive cases exhibit a highly restricted pattern of expression of latent gene products, including only EBNA1 and the EBERs (Latency I). However, it has been recently reported that some cases, in addition to EBNA1 and the EBERs, also express EBNAs 3A, 3B, and 3C, but still lack EBNA2 and the latent membrane proteins (Kelly GL, 2006). This peculiar restricted latency pattern has stimulated an intense and still ongoing debate about the role of EBV in the pathogenesis of Burkitt lymphoma. Certainly, EBNA1 plays a crucial role in the maintenance and replication of the viral genome, but its oncogenic potential is still highly controversial (Young LS, 2004; Wilson JB, 1996). As the EBERs are believed to possess anti-apoptotic activities, as well as the ability to induce the expression of IL-10, which may promote cell growth and survival, it has been suggested that these RNAs may play an essential role in the oncogenesis of Burkitt lymphoma (Kitigawa N, 2000). Moreover, analysis of the chromosome breakpoints in the myc-activating translocations indicates that these translocations have occurred during either somatic mutation or class switch recombinations, which are both unique processes of germinal centre cells (Küppers R, 2001).
- *Hodgkin's lymphoma (HL)* is the most common EBV-associated lymphoma in the US and Europe. About 40% of Hodgkin's lymphomas are EBV-associated (Küppers R, 2003). Interestingly, in the tumor tissue only a small subset of cells, the so-called Hodgkin–Reed-Sternberg (HRS) cells, are the EBV-transformed tumor cells, primarily of B cell origin, in the tumor tissue (Kutok JL, 2006), while the majority of cells

composing the tumor mass are infiltrating lymphocytes (TILs). This indicates that HL has already managed to generate an immunosuppressive environment that allows tumor cells to grow despite extensive homing of immune cells to the tumor site. HRS cells have been indeed shown to produce immunosuppressive cytokines, including IL-10, IL-13 and TGF-β (Herbst H, 1996; Hsu SM, 1993). Therefore, HRS cells employ several mechanisms allowing escape of the tumor from immune recognition. As a result, regulatory T (Treg) cell populations are enriched in HL tissues. Among the CD4+ Treg cells are IL-10 producing cells and CD4+CD25+ natural Treg cells (Marshall NA, 2004), able to suppress peripheral blood cell proliferation and cytokine secretion. Therefore, regulatory T cell populations may suppress EBV-specific immune control locally. In addition to this local immune suppression, selective systemic impairment of EBV-specific T cell responses might also contribute to HL development. Along these lines, HL patients have diminished EBNA1 specific CD4+ T cell responses, while they maintain CD8+ T cell responses against other latent and lytic EBV antigens (Heller KN, 2008). These findings suggest that immunotherapeutic approaches should be developed to correct both the selective systemic immune impairment and tumor microenvironment specific deficiencies in EBV-specific immune control. Since the tumor cells seem to have no defects in antigen processing for MHC class I (MHC-I) presentation, interventions to correct the selective systemic loss of EBV specific T cell responses and to overcome the local immune suppression in the tumor tissue should be explored as treatments of this EBV-associated malignancy and ideally such modalities could be used for prevention in high risk populations.

- *Lymphomatoid granulomatosis (LG) is a* rare systemic lymphoid tumor that mainly affects skin, brain, lungs, and kidneys. EBV EBERs have been detected in nearly all LG cases affecting the lung, which contain large B cells and a florid T cell reaction, but is absent from T cell or indeterminate lymphomas presenting as a LG. Similarly, only cutaneous LG with B cell monoclonal populations carry the virus (Myers JL, 1995). This diagnosis is rarely established clinically and lesions can show extensive necrosis that render diagnosis difficult. There is limited information about the pattern of EBV protein expression in LG, but in one study all three EBV-positive LG cases investigated expressed LMP1, two of which were also EBNA2 positive. EBV detection in these lesions can be very helpful for diagnostic purposes.

- *Angioimmunoblastic T cell lymphoma (AITL)* is characterized by a polymorphic cellular infiltrate including T and B cells, hyperplastic small vessels and a variable degree of follicular disorganization. The majority of AITL cases (81–95%) contain a variable number of EBV EBER-positive large B blasts but also some EBV-positive T cells (Zhou Y, 2007). Interestingly, EBV infection associated with expansion of infected B cells is a feature of AITL, but not of unspecified peripheral T-cell lymphomas, which are all EBV-negative. The detection of the virus can therefore contribute to the diagnostic process.

Tumors derived from T and NK cells. The rate of association of the virus with these tumors is so high that the absence of the virus virtually excludes diagnosis. All described entities, mostly encountered in Japan and South-East Asia, are closely related and can also arise in a single patient.

- *Peripheral T cell lymphoma, NK tumors and EBV-associated haematophagocytic syndrome (HS):* HS is frequently observed in infants with immune deficiencies and primary or chronic EBV infection is one of its well recognized aetiologies. In this case, T lymphocytosis and EBV-positive T cells are frequently observed in the blood and tissues. Affected individuals are at high risk of subsequent or concurrent development of EBV-positive T-cell lymphomas and NK cell tumors (Rickinson AB, 2007). Detection of EBV in either normal T lymphocytes or tumor cells establishes the diagnosis. Cancer cells express EBER and also LMP1.

- *NK leukaemias* are mainly observed in adolescents of the Far East in association with hepatosplenomegaly and multiple cutaneous lesions (Gatter K, 2002). Histological examination identifies large granular lymphocytes expressing NK markers. The rate of EBV association with these lesions approaches 100%.

- *Extranodal NK/T cell lymphoma*, nasal type consists of an angio-invasive and angio-destructive lymphoid infiltrate with necrosis that affects and can cause destruction of the nasal cavity and of several anatomical structures of the mid-face (Al-Hakeem DA, 2007). These tumors arise most commonly in South-East Asia but also in Central and South America. EBV is present in virtually all cases (EBER positive, EBV proteins rarely expressed, but in this case a more or less complete latency II pattern is observed). Patients with this tumor can develop EBV-associated HS.

- *Inflammatory pseudo-tumor-like follicular dendritic cell tumor (IPLFD)* preferentially develops in liver and spleen, or more rarely in lymph nodes. This exceptional tumor is characterized by a prominent inflammatory background including numerous lymphocytes and plasma cells, among which neoplastic follicular dendritic cells (FDC) can be identified (Cheuk W, 2001). Viral infection is readily established with an EBER assay showing intense signals in all cancer cells; LMP1 staining, although consistently positive, is generally weak. Due to the consistent association with EBV, diagnosis of this entity requires detection of the virus.

EBV AND NASOPHARYNGEAL CARCINOMAS (NPC)

Nasopharyngeal carcinoma is classified as a malignant neoplasm arising from the mucosal epithelium of the nasopharynx, most often within the lateral nasopharyngeal recess or fossa of Rosenmüller. There are three histopathologic subtypes of NPC: a well-differentiated *keratinizing* type, a moderately-differentiated, *non-keratinizing* type, and an *undifferentiated* type, which typically contains large numbers of non-cancerous chronic inflammatory lymphocytes. The undifferentiated form is the most common and also the most strongly associated with EBV infection. NPCs are rare in most countries, especially in Europe and North America (incidence below 1/100,000). However, it has a high incidence in several areas in Southern China, especially in the Cantonese region around Guangzhou, where the incidence is approximately of 30–80/100,000 cases per year. Other areas of high incidence include Taiwan, Vietnam and the Philippines (Wei WI, 2005), where the diet probably plays a carcinogenic role, being composed of salt-cured fish and meat, and when such food is cooked volatile nitrosamines are likely to be released. Since epithelial infection by EBV can be demonstrated in vitro (Borza CM, 2002), but it has not been convincingly documented in vivo, the etiology of this tumor is still quite mysterious. Generally, vigorous humoral and cellular immune responses control the proliferation of EBV-infected cells in healthy virus carriers. Indeed, both non-specific (NK-cell mediated) and EBV-specific (T-cell mediated) responses were shown to play important roles during primary infection, while EBV-specific T cells appear to be critically involved in restraining the proliferation of EBV infected cells during life-long persistent infection. On these grounds, recent studies have confirmed that T cells specific for EBV antigens expressed during latent and productive infection are maintained in the blood of healthy carriers at relatively high frequencies throughout life

(Hislop AD, 2002). Moreover, direct evidence for the importance of these EBV-specific T cells in controlling the oncogenic capacity of the virus is provided by the occurrence of EBV-associated immunoblastic lymphomas in the patients where their activity is impaired by congenital immunodeficiency, immunosuppressive therapy or HIV infection (Levine AM, 1994). Thus, these EBV-associated lymphomas can be prevented or even cured by adoptive transfer of in vitro activated and expanded EBV-specific T cells (Heslop HE, 1996; Burns JM, 2006), suggesting that reconstitution of EBV-specific immunity could also be a useful strategy in the management of NPCs. Indeed, it has been clearly documented that EBV is present in 100% of NPCs and establishes a latency pattern with EBNA1 and LMP1 or LMP2 protein expression. Early after the discovery of EBV association with NPC, a deregulation of the EBV-specific immune response with elevated IgA titers against the virus was noted (Henle G, 1976). This indicated that the immune response at the site of tumor development was changed, and that the tumor might influence its microenvironment to facilitate growth. Indeed, recent studies supported the notion that local immune suppression rather than a systemic deficiency in EBV-specific immune control may contribute to NPC development. In these studies, EBV specific CD4+ and CD8+ T cell responses could be reactivated from peripheral blood of NPC patients (Lin X, 2007; Li J, 2007). Even though LMP1 and LMP2 specific CD8+ T cells were enriched in tumor infiltrating lymphocytes, their cytotoxicity and cytokine secretion was impaired. This impairment could be due to the presence of CD4+CD25+FoxP3+ natural Treg cells in the tumor tissue, which could suppress EBV-specific immune responses against NPC even after correct homing of effector T cells (Lau KM, 2007). In addition to active T cell suppression at the tumor site, the efficiency with which NPC can present antigens to T cells might also be compromised. While earlier studies based on a limited number of NPC cell lines suggested that antigen processing for MHC-I presentation was intact in NPC cells (Khanna R, 1998), a more recent study on primary tumor tissues suggested that the MHC-I antigen processing machinery is down-regulated in the majority of tumors (Ogino T, 2007). Even though no functional deficiency of MHC-I antigen presentation could be tested in this later study, this makes possible to speculate that in addition to active immune suppression at NPC tumor site, the recognition of tumor cells by CD8+ T cells could be also impaired. Together, these data suggest that NPC impairs EBV-specific immune control locally, while allowing efficient systemic immune responses against this virus.

Conventional treatments for NPC frequently fail and are often accompanied by severe long-term side effects (Li J, 2007). This, together with the regular association of NPC with EBV infection and the expression of immunogenic viral antigens in the tumor cells, has stimulated intense efforts to develop strategies of immune intervention that could complement or even substitute current therapeutic regimens for a better control of NPC.

IMMUNOTHERAPY APPROACHES AGAINST EBV-ASSOCIATED MALIGNANCIES

The development of malignancies associated with EBV is largely favored by an underlying defect in EBV-specific cytotoxic T lymphocyte (CTL) immunity and function. Much work has been focused in the last years on the reconstitution of CTL immunity to EBV in transplant patients, who are rendered susceptible to PTLD by iatrogenic immune suppression modalities. Moreover, recent data indicates that other EBV-associated diseases such as NPC, HD, BL, and chronic active EBV infections (CAEBV) can potentially be treated with immunotherapeutic approaches (Fig 3).

To date, there are only limited experiences with human EBV vaccines (Gu S.Y., 1995; Khanna R, 1999). Although potentially ideal for preventing EBV-associated malignancies, vaccines providing life-long immunity against primary EBV infection may not be feasible, because the type of immunity required to prevent repeated infection through mucosal surfaces is not clearly defined. Moreover, repeated infections with different EBV strains have been described, suggesting that the natural immune response to EBV is not sufficient to protect healthy EBV-positive individuals from recurrent infections. Nevertheless, it was clearly demonstrated that the risk of developing HL is 4-fold higher after resolution of IM, which is symptomatic of primary EBV infection and then of a massive expansion of EBV-specific T cells (Hjalgrim H, 2003). Therefore, preventive vaccination to avoid uncontrolled virus replication and successive “scaring” of the immune system could decrease the incidence of EBV-associated malignancies. Vaccine strategies for the immunotherapy of EBV-related tumors should seek to elicit or boost specific cellular immune response against EBV antigens expressed in these malignancies. Individuals likely to benefit from this approach are EBV-

seronegative patients prior to solid organs transplant (SOT) or patients affected by EBV-associated malignancy with a low tumor burden or in remission. However, vaccine strategies are unlikely to be the optimal method to enhance EBV-specific T-cell responses for patients who are immunocompromised due to immunosuppressive therapies after transplantation or as a result of HL. Thus in such cases, *the adoptive immunotherapy* with ex vivo activated EBV-specific CTL seems to be more promising, especially because it allows genetic modifications of T cells able to enhance their function.

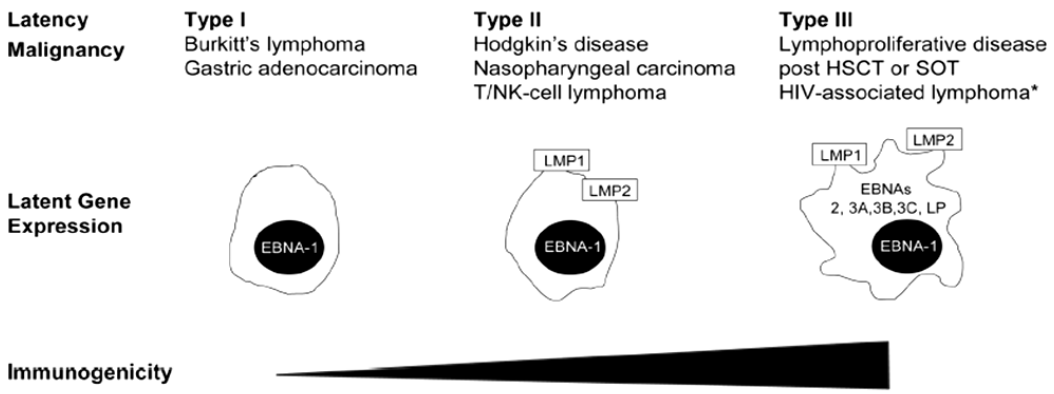

Figure 3. EBV latent protein expression and immunogenicity of common EBV-associated malignancies. *Only EBV latent protein expression is shown. In EBV latency types I-III EBV encoded-RNAs (EBERs) and the BamHI-A rightward transcripts (BARTs) are also expressed.*

1. *EBV-specific immunotherapy in PTLDs.* EBV infection poses a significant problem in transplant patients who are greatly immunosuppressed in order to prevent chronic organ rejection. Risk factors for the development of PTLD include EBV-seronegativity in the transplant recipient, the type of organ transplanted (highest in lung and heart and lowest in liver and kidney), and the level and type of immune suppression. PTLD emerges as either of recipient or donor origin, depending on the type of transplant. For example, bone marrow transplant (BMT) patients develop PTLD of donor origin, as EBV-infected B cells derived from the donor marrow proliferate uncontrollably into lymphoma. Conversely, solid organ transplant (SOT) patients develop PTLD of recipient origin, as EBV released from the transplanted organ infects the recipient's B cells. On these grounds, initial studies investigated the potential of EBV-specific CTLs to treat PTLD in BMT patients, as CTLs could be

easily generated from EBV-seropositive, immunocompetent donors. Pioneering studies (Heslop HE, 1994; Rooney CM, 1995) demonstrated that PTLD was resolved after adoptive transfer of EBV-specific CTLs grown from donor peripheral blood mononuclear cells. The method developed to stimulate and expand large numbers of EBV-specific CTLs utilized the donor's autologous EBV-immortalized lymphoblastoid B-cell lines (LCLs), which were co-cultured with donor PBMCs in the presence of interleukin-2 (IL-2). Similar to PTLD tumor cells, LCLs also have a latency III phenotype and can activate polyclonal EBV-specific CTLs with a broad reactivity to a range of EBNA-derived p epitopes. The resulting EBV-specific CTLs used in these studies killed donor LCLs in vitro, did not compromise allograft function, and most importantly, eradicated tumors.

2. *EBV-specific immunotherapy in NPC and HL*. In addition to these preventive vaccines, therapeutic immunizations against EBV-associated malignancies are also being pursued. Clinical evidence accumulated so far indicates that adoptive therapy with EBV-specific CTLs (EBV-CTLs) is safe, well tolerated and particularly effective in the case of most immunogenic tumors like PTLD (Leen AM, 2008). In latency II EBV-associated malignancies, however, the more restricted pattern of viral latent antigen expression strongly limits the therapeutic potential of EBV-CTLs obtained by conventional protocols based on the use of autologous LCLs as a source of viral antigens. In fact, the infusion of EBV-targeted autologous CTLs was shown to enhance specific immune responses and to induce objective clinical responses only in a proportion of NPC and HL cases (Lin CL, 2002; Bollard CM, 2004). This is probably due to the weak immunogenicity of LMP1 and LMP2, To improve protocols for *in vitro* expansion of T cells specific for the EBNA1, LMP1 and LMP2 antigens, which are present in these malignancies, recombinant viruses encoding for these EBV products have been utilized to expand specific CD8+ T cells, which could protect against LMP-positive tumor growth in mice (Smith C, 2006; Duraiswamy J, 2004). However, these T cell lines, targeting a select subset of EBV antigens, are just now starting to be tested in patients. As an alternative to passive immunization, adoptive T cell transfer of EBV antigen loaded DCs has been evaluated for inducing protective CD8+ T cell responses against NPC. Although LMP2-specific CD8+ T cells could be expanded after peptide-pulsed DC injection in NPC patients, these responses were too weak or transient . Thus, vaccine approaches that primarily target CD8+ T cells have

not yielded sufficient therapeutic success against EBV-associated lymphomas. Learning from these trials and as a result of a better understanding of the crucial role for CD4+ T cells in assisting CD8+ T cell immunity, more recent vaccine formulations aim to incorporate both CD4+ and CD8+ T cell antigens. In addition to CD4+ T cell help for CD8+ T cell responses, CD4+ T cells can also target EBV-transformed B cells directly, adding to their value as vaccine targets. As previously observed, many of these immunization strategies target DCs, which have been shown to be more efficient than LCLs in expanding EBV specific T cells and are capable of priming protective CD4+ and CD8+ T cell responses against EBV transformed B cells *in vitro* (Bickham K, 2003). CD4+ and CD8+ T cells, expanded with DCs, which had been infected with a recombinant adenovirus encoding LMP2, were able to kill NPC cells (Pan Y, 2006). Finally, considering that NPC's and HL's malignant cells have functional antigen processing machinery and express HLA and co-stimulatory molecules (Khanna R, 1998; Lee SP, 2000), the demonstration that other viral latent proteins expressed by these neoplastic cells may serve as tumor-associated antigens could provide the rational background to improve the clinical efficacy of adoptive immunotherapy protocols in this setting.

Adoptive immunotherapy with EBV-specific CTLs has proven to be an effective strategy in many PTLDs to (Rickinson AB, 2007) reconstitute EBV-specific immunity, (ARC, 1997) prevent the development of EBV-PTLD (Tao Q, 2006) and treat patients with established EBV-PTLD. For other EBV-associated malignancies, the use of EBV-specific CTL has proven less efficacious; however the results obtained so far are sufficiently encouraging to justify continued active exploration of this approach. Novel approaches are being developed to enhance the potency of EBV-specific immunotherapy by targeting CTL to subdominant EBV proteins and by genetically modifying these effector cells to render them resistant against inhibitory cytokines or immunosuppressive therapies. Notably, such strategies could have broad implications for the adoptive immunotherapy of a broader spectrum of human cancers with defined tumor antigens. All these approaches open promising avenues to enhance or prime protective EBV-specific immune responses, which have been suppressed by the tumor cells itself or by their microenvironment, and whose absence might predispose for the development of EBV-associated malignancies.

BARF1 AS TARGET FOR IMMUOTHERAPY

While EBV-specific CTLs have been used successfully for the prophylaxis and treatment of the highly immunogenic PTLDs, the clinical experience for other EBV-associated malignancies, such as HL and NPC, is limited and the results obtained so far indicate that EBV-specific CTLs are less effective in these settings. Decreased CTL efficacy most likely reflects immune evasion strategies by tumor cells, including down-regulation of immunodominant EBV proteins and local secretion of inhibitory cytokines. One of the possible approaches to overcome these limitations is the identification of additional viral proteins expressed by tumor cells and that may serve as tumor-associated antigens to be targeted by improved CTL induction and expansion protocols.

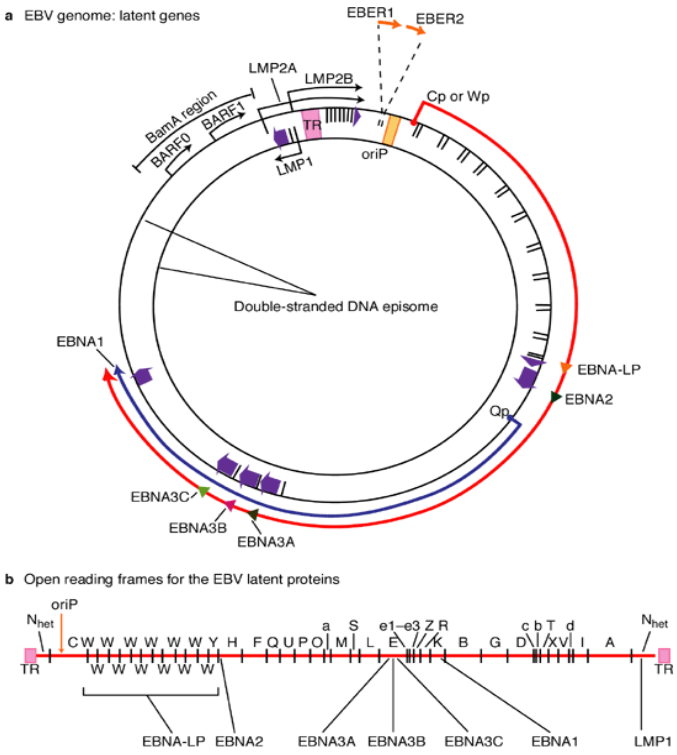

Figure 4 .The Epstein–Barr virus genome. (a) *Diagram showing the location and transcription of the EBV latent genes on the double-stranded viral DNA episome. Transcripts from the BamA region can be detected during latent infection and here are shown the locations of the BARF0 and BARF1 coding regions.* ***(b)*** *Diagram showing the location of ORFs for the EBV latent proteins on a BamHI restriction endonuclease map of the prototype B95.8 EBV genome.*

The BamHI-A fragment of the EBV genome encodes for the BARF1 gene, located at nucleotide positions 165449-166189, of the B95.8 strain (Fig 4). The BARF1 gene is translated into a 221 amino acids long protein, with a calculated mass of 31-33 kDa (Zhang CX, 1988). This protein may play different functions in immunomodulation and oncogenicity. In particular, it has been demonstrated that BARF1 functions as a soluble receptor for human colony-stimulating factor 1 (hCSF-1) (Strockbine LD, 1998), and recombinant BARF1 inhibits the ability of hCSF-1 to induce proliferation of bone marrow macrophage progenitor cells. Notably, hCSF-1 is known to have a number of other activities, including induction of mononuclear cells to release cytokines, such as interferon alpha (IFN-α), tumor necrosis factor alpha (TNF-α), granulocyte colony-stimulating factor (G-CSF), and IL-1 (Roth P, 1992). Thus, the ability of BARF1 to block hCSF-1 activity might impair cytokine release from mononuclear cells, thereby reducing cellular immune response to EBV. BARF1 could also act as an oncogene when stably expressed in mouse fibroblasts and monkey kidney cells (Wei MX, 1994; Wei MX, 1997), being able to induce the expression of the c-*myc* proto-oncogene and the CD21 and CD23 B-cell activation antigens (Wei MX, 1994). Interestingly, BARF1 was found in EBV-immortalized epithelial cells, without the expression of LMP1, which is essential for B-cell immortalization (Danve C, 2001) and was also capable of inducing malignant transformation in Balb/c3T3 cells (Wei MX and Ooka T, 1989) and in human Louckes and Akata B-cell lines (Wei MX, 1994). Moreover, Cohen and colleagues (Cohen JI, 1999) showed that both recombinant and EBV-derived BARF1 protein were able to inhibit IFN-α production by human monocytes. Therefore, BARF1 might also play an important role in modulating the innate host response to promote survival of virus-infected cells *in vivo*. Although BARF1 is thought to be a lytic gene in B-lymphocytes, since it is not expressed in BL cell lines (Zhang CX, 1988), its expression was detected in NPC and EBV-positive gastric carcinoma (GC) tissues in the absence of the expression of other lytic genes (Seto E, 2005). This suggests that BARF1 may be expressed as a latent gene in EBV-associated epithelial malignancies. Notably, computer analysis of BARF1 sequence predicted a cleavage site after the 20th N-terminal amino acid. The secretion of a 29 kDa BARF1-coded polypeptide from human B cells was already reported by Strockbine et al. (Strockbine LD, 1998), showing that almost all BARF1 protein is secreted in culture medium rendering its detection difficult in intracellular compartments. Thus, one possible mechanism of oncogenic transformation induced by BARF1 might be autocrine/paracrine cell cycle activation by the secreted form of its translation product. Finally, BARF1 is also able to induce humoral

responses in EBV-seropositive individuals and may serve as a target for antibody-dependent cellular cytotoxicity in NPC patients (Tanner JE, 1997).

AIMS OF THE STUDY

In the last years, strong evidence indicates that adoptive therapy with EBV-specific CTLs (EBV-CTLs) is safe, well tolerated and particularly effective in the case of most immunogenic tumors like PTLD (Merlo A, 2008; Leen AM, 2008). Nevertheless, in NPC and HL malignancies the more restricted pattern of viral latent antigen expression (LMP1 and LMP2) strongly limits the objective clinical responses only to a low proportion of cases (Lin CL, 2002; Comoli P, 2005; Strathoff KC, 2005; Bollard CM, 2004; Rickinson AB, 1997). In this regard, the demonstration that other viral latent proteins expressed by these tumor cells may serve as tumor-associated antigens could provide the rational background to improve the clinical efficacy of immunotherapy targeting latency II EBV-associated malignancies.

The present study was aimed at characterizing the immunogenic properties of the BARF1 protein with the final goal to verify its potentially usefulness as target to improve the efficacy of EBV-based immunotherapy for NPCs. On these grounds, we have investigated the immunogenicity of BARF1 in Italian NPC patients, and identified new BARF1 epitopes presented in the context of common HLA class Ialleles. We also demonstrated that BARF1-specific and highly effective CTLs can be easily obtained from EBV-seropositive donors, an important pre-requisite for adoptive immunotherapy. These promising results provide a strong rationale to design BARF1-based vaccines for active immunotherapy approaches of EBV-related disorders and, interestingly, may also allow the development of improved protocols of adoptive immunotherapy based on the use of EBV-CTLs enriched in BARF1-specific effectors.

MATERIALS AND METHODS

NPC PATIENTS AND HEALTHY DONORS

Eighteen tumour samples, diagnosed according to histologic criteria provided by W.H.O. classification (Henle G, 1976), 10 biopsies of normal nasopharyngeal mucosa, and 10 blood samples were obtained from NPC patients. All NPC cases investigated were EBV-associated as shown by *in situ* hybridization for EBERs. Buffy coats from 11 EBV-seropositive healthy donors were also collected and included in present study. Peripheral blood mononucleated cells (PBMCs) were isolated on Ficoll density gradients and cryopreserved immediately. All specimens were obtained after informed consent from both patients and donors. HLA-A and -B typing was performed in all cases by sequence-based typing, according to standard high-resolution typing techniques.

CELL LINES AND CULTURE CONDITIONS

The following human cell lines were used in this study: the Akata and Raji Burkitt's lymphoma cell lines, the Granta 519 mantle cell lymphoma cell line (HLA-A2), the DAA3 EBV-transformed B lymphoblastoid cell line, and the transporter associated with antigen-processing-deficient T2 cells transfected with the HLA-A*0201 gene (T2-A2). EBV-transformed LCLs were generated *in vitro* by transformation of B cells using the standard EBV isolate B.95.8. All cell lines were cultured in RPMI-1640 (Gibco, Grand Island, NY), containing 10% fetal bovine serum (Gibco), 2 mM L-glutamine, 100 μg/ml streptomycin and 100 IU/ml penicillin (Sigma), with the exception of Granta cell line, which was cultured in complete Dulbecco's Modified Eagle's Medium (DMEM, Cambrex Bio Science Walkersville, Inc., MD). Induction of the EBV lytic cycle was achieved by incubation of cells with a combination of 12-O-tetradecanoyl-phorbol-1-acetate (Sigma, 20 ng/ml final concentration) and sodium butyrate (Sigma, 3 mM final concentration) in standard medium for 48 h.

ANALYSIS OF BARF1 mRNA EXPRESSION

Total RNA was extracted from cell lines and UNPC tumor tissues with Trizol reagent (Invitrogen, Carlsbad, CA), and then treated with DNase I (Promega, Milan, Italy). Reverse transcriptase PCR (RT-PCR) was carried out as described (Lin X, 2007). Briefly, 1 microgram of total RNA was used for a first-strand cDNA synthesis in a 20 μl reaction volume using 0.5 μg of random primers. Reverse-transcription was done with AMV-reverse transcriptase (Promega) according to manufacturer's instructions. cDNA aliquots were then subjected to PCR analysis using primer pairs specific for BARF1 and glycealdehyde-3-phosphate dehydrogenase (GAPDH) (Sigma, Milan, Italy). Sequences of primer pairs were as follows, BARF1: 5'-GGCTGTCACCGCTTTCTTGG-3' and 5'-AGGTGTTGGCACTTCTGTGG-3'; GAPDH: 5'-GCCTCCTGCACCACCAACTG-3' and 5'-CGACGCCTGCTTCACCACCTTCT-3'. Amplification conditions were: denaturation step at 94°C for 3 min, annealing at 60°C for 1 min, extension at 72°C for 1 min for 30 cycles and a final extension step at 72°C for 5 min.

PEPTIDE-PREDICTION ANALYSIS, BIOINFORMATICS AND PEPTIDE SYNTHESIS

The BARF1 protein sequence spanning the first 60 amino acids and including the conserved transforming domain was analyzed by immuno-informatic tools to identify candidate antigenic epitopes presented by the HLA-A*0201 allele. Peptides were selected on the basis of their predicted binding affinity to HLA-A*0201 according to SYFPEITHI (www.syfpeithi.de) and BIMAS (www-bimas.cit.nih.gov/molbio/hla_bind/) prediction softwares available on the web. The HLA-A*0201-restricted Flu matrix 1 ($M1_{58-66}$) peptide (GILGFVFTL) was used as positive control. All peptides were synthesized by fluorenylmethoxycarbonil synthesis (Primm, Milan, Italy) and purity (>95%) was determined by reverse-phase high-performance liquid chromatography and verified by mass spectral MALDI-TOF analysis. Peptides were dissolved in DMSO at a concentration of 2.5 mg/ml and stored at -70°C until use. Work stocks for each peptide were prepared in phosphate-buffered saline at a final concentration of 500 μg/ml and stored frozen. Immuno-informatic prediction allowed also the identification of 3 HLA*A1101, 2 HLA-A*2402, and 5 HLA-B*5101 candidate BARF1 epitopes.

The predicted binding affinity of possible "variant" BARF1 HLA-A*0201 epitopes carrying single amino acid changes in the anchor sites will be analyzed in comparison with that of reference epitopes (Küppers R, 2003) using the SYFPEITHI (www.syfpeithi.de) (Hsu M, 1993), BIMAS (www-bimas.cit.nih.gov/molbio/hla_bind/) (Marshall NA, 2004), and NetMHC (www.cbs.dtu.dk/services/NetMHC/) (Heller KN, 2008) prediction software available on the web. The immunogenicity of the putative "variant" epitopes identified will be also investigated at the functional level in comparison with that of the "native" epitopes using synthetic peptides in the T2-A2 stabilization assay and IFNγ-ELISPOT assay (Küppers R, 2003). This latter will be carried out with PBMCs of both EBV-seropositive donors and UNPC patients. We also plan to complete the HLA class I genotyping of a relatively large series of Italian UNPC. This will be accomplished by performing a DNA polymerase chain reaction (PCR) sequencing based typing with primers specific for both locus A and B (Myers JL, 1995). Additional predicted BARF1 epitopes, presented in the context of HLA alleles different from HLA-A*0201 and not under-represented in Italian UNPC, will be identified using the same immuno-informatic engines. Validation of these additional BARF1 epitopes will be carried out with the same protocols used for the HLA-A*0201 epitopes (Küppers R, 2003). The results obtained will allow the selection of the most suitable BARF1 peptides for the synthesis of MHC-peptide tetrameric complexes. These reagents will be used in the context of the HLA class I profile of Italian UNPC to set up and validate assays to monitor BARF1-specific CD8+ T cell responses at the epitope level. If the validation of the predicted epitopes will be unsuccessful, overlapping peptide pools spanning the entire BARF1 amino acid sequence will be screened by IFN-γ ELISPOT for their ability to elicit CD8+ T cell responses in a panel of healthy donors with the appropriate HLA class I background.

MHC STABILIZATION ASSAY

Selected peptides were tested for their ability to bind to HLA-A*0201 molecules in a MHC-class I stabilization assay using the T2-A2 cell line. Briefly, T2-A2 cells (2×10^5) were pulsed with 20 μg/ml peptide and 5 nM β_2-microglobulin (Chemicon, Milan, Italy), then incubated at 26°C for 16 h, followed by 3 h at 37°C. HLA-A*0201 expression was then measured using the mouse anti-human HLA-A2, -A28 PE-conjugated BB7.2 monoclonal antibody (Acris Antibodies GmbH, Hiddenhausen, Germany) or the pan-HLA Class I monoclonal antibody W6/32 (Dako, Milan, Italy), followed by incubation with a PE-conjugated polyclonal goat

anti-mouse antibody (Dako). The fluorescence index (FI) for each peptide was calculated according to the following formula: FI = mean fluorescence intensity (MFI) of T2-A2 cells + peptide / MFI of T2-A2 cells without peptide. The Flu M1 p58-66 peptide was used as positive control and the background expression of HLA-A2 was determined using DMSO as a negative control. Peptides were considered to stabilize HLA-A2 molecules with high affinity when the FI was ≥ 1.5 and low affinity when the FI was between 1.1 and 1.49. All the assays were repeated a minimum of three times, and the results are given as means of replicate experiments.

EXPRESSION AND PURIFICATION OF RECOMBINANT BARF1 PROTEIN

The p29 BARF1 protein was expressed in 293-T cells using a recombinant adenovirus system as already described (Pritzker KP, 1970). After 24 hours infection, cells were washed four times with PBS, then seeded in 4 ml DMEM without serum and phenol, and further incubated for 36 to 48 h. After harvesting, the cells were centrifuged at 2000 g for 20 min. Recombinant adenovirus and cell debris were eliminated by ultracentrifugation at 167000 g for 2 h. The culture medium was concentrated using Ultrafree-15 filter device (Millipore). Concanavaline A affinity columns were used to purify BARF1 p29 protein from the concentrated medium. Briefly, the Concanavaline A was diluted in a buffer containing 1M NaCl, 0.1M (Na_2HPO_4, NaH_2PO_4) at pH 6, 10^{-3}M ($CaCl_2$, $MgCl_2$, Mn Cl_2) and centrifuged for 15 min at 3000 g. Concentrated medium was then incubated for 18 h with Concanavaline A. After four washes with the same buffer, p29 protein was eluted with 1M MGP (Methyl α-D-glucopyranoside) for 12 h.

IFN-γ ELISPOT ASSAY

The IFN-γ release ELISPOT assay was performed using a commercial kit (Human IFN gamma ELISPOT kit, Endogen, Tema Ricerca, Bologna, Italy) according to manufacturer's instructions. The assay was carried out using either 15 μg/ml BARF1 protein-pulsed monocytes as stimulators (5 x 10^4 cells/well) and purified CD4+ or CD8+ cells as responders (5 x 10^4 cells /well), or monocytes pulsed with 20 μg/ml peptide and isolated CD8+ T lymphocytes as responders. Autologous monocytes were obtained from PBMC by plastic

adherence. For the evaluation of memory responses against BARF1 protein, monocytes were co-cultured over-night with recombinant p29 BARF1 protein (15 μg/ml) and the BioPORTER reagent QuickEase™ (Gene Therapy Systems, Inc. San Diego, CA). Peptide-loaded monocytes were generated by re-suspending the cells in RPMI 1640 10% human AB serum, supplemented with 10 μg/ml of human β_2-microglobulin and aliquoting monocytes into ELISPOT assay plates. Peptides were added at a final concentration of 20 μg/ml and plates were incubated 2 h at 37°C and 5% CO_2. Purified effectors were obtained by immunomagnetic enrichment protocols using human CD4+ or CD8+ T cell isolation kit II (Miltenyi Biotec, Calderara di Reno, Italy), and were added to protein or peptide-loaded monocytes at the effector:target ratio of 1:1. Monocytes stimulated with tetanus toxoid from *clostridium tetani* (TT, 5 μg/ml, Calbiochem, San Diego, CA) or FLU $M1_{58-66}$ peptide (20 μg/ml) were used as positive controls and un-stimulated monocytes as negative control. Cells were seeded onto ELISPOT capture plates in quadruplicates and incubated for 48 h at 37°C and 5% CO_2. All plates were evaluated by a computer-assisted ELISPOT reader (Eli.Expert, A.EL.VIS GmbH, Germany). The number of spots in negative control wells (range of 0–5 spots) was subtracted from the number of spots in stimulated wells. Responses were considered significant if a minimum of five IFN-γ producing cells were detected in the well.

AUTOLOGOUS DENDRITIC CELL GENERATION AND PRODUCTION OF PEPTIDE-SPECIFIC CTLS

Purified PBMCs were re-suspended in serum-free medium and incubated at 37°C to allow for plastic-adherent step. After 1h, non-adherent peripheral blood lymphocytes were removed and vitally cryopreserved, while immature dendritic cells (DC) were obtained by culture adherent monocytes in complete RPMI 1640 supplemented with 10% of heat-inactivated healthy donors AB serum, recombinant human GM-CSF (50 ng/ml) and IL-4 (25 ng/ml), both from R&D Systems (Abingdon, UK). Cells were substituted with 10% of fresh medium and cytokine concentrations were re-established on days 3 and 6. On day 6, DC maturation was also induced with LPS (1.5 μg/ml) from Salmonella Typhimurium (Sigma). After over-night incubation at 37°C, 10^6 mature DC/ml were seeded in 48-wells plates and pulsed respectively with the p2-10, p23-31 and p49-57 BARF1 peptides (10 μg/ml). Three μg/ml of human purified β2-microglobulin were added (Chemicon) and then incubated for 2 hours at 37°C in

5% CO_2. Aliquots of peptide-pulsed DC were vitally stored frozen and used in weekly CTL re-stimulations, while aliquots of 10^5/ml DC were re-suspended in complete medium, supplemented with rhIL-7 (20 ng/ml, R&D Systems) and co-cultured in 24-wells plates with autologous peripheral blood lymphocytes, at 10:1 or 20:1 effector:target ratio. rhIL-2 (3 ng/ml, R&D Systems) was added on day 2, and every 4 days thereafter, to all cultures. After three/four re-stimulation with peptide-pulsed DC, CTLs were tested for cytotoxic activity.

STANDARD CALCEIN-AM RELEASE ASSAY

Cytotoxic activity of peptide-specific CTLs was evaluated using autologous LCLs and the EBV+, BARF1+ Granta 519 cell line as targets in a calcein–AM (ca-AM) release assay. Target cells ($2x10^5$) were re-suspended in 1 ml of Hanks Balanced Salt Solution without phenol red (HBSS), supplemented with 1% FCS, labelled with 7.5 μM of ca-AM (Calbiochem) and incubated 30 min at 37°C 5% CO_2. Labelled cells were washed three times and seeded in 96-wells plate at a concentration of 5 x 10^3 cells/well. CTLs were added at 20:1, 10:1, 5:1, 2.5:1 and 1.25:1 effector:target ratio. All tests were performed in triplicate. The HLA-A*0201-specific mAb cr11.351 was added to the target cells and incubated at room temperature for 30 min to assess the HLA-A*0201 restriction of CTL responses. To obtain total calcein-releasing cells, targets were incubated with 100 μl/well of lysis buffer (25 mM sodium borate, 0.1% Triton-X100 in HBSS, pH 9.0). Spontaneous release was determined by seeding target cells in 100 μl/well of HBSS. Plates were incubated 4 h at 37°C and 5% CO_2 in a volume of 200 μl/well. Following incubation, the content of each well was mixed, plates were centrifuged and 100 μl of the supernatant was transferred to a 96-well black culture plate. Fluorescence intensity was measured by reading the plates from the top using a SpectraFluorPlus (Tecan, Austria). Excitation and emission filters were 485 and 535 nm, respectively and gain was set at 70. The % of lysis was calculated as follows:

$$\text{Lysis \%} = \frac{\text{Experimental release - Spontaneous release}}{\text{Total release - Spontaneous release}} \times 100$$

GENERATION OF ENGINEERED BARF1+ CELL LINES AND IN VIVO STUDIES

Ectopically and stably expression of the HLA-A*0201 allele were induced using the pBABE retroviral vector (Addgene, Cambridge) in EBV+ c.666.1 cell line, derived from UNPC patients. This strategy has also been successfully used to force the expression of the HLA-A*0201 allele in the DG75 cell line that profoundly down-regulates HLA class I upon *in vivo* transplantation (unpublished observation). As additional models of BARF1+ lymphoid tumors, we also used: the EBV-negative DG75-A2 cell line ectopically expressing BARF1 through retroviral infection with the pLXSN retroviral vector (Clontech, CA, USA), the EBV+ HLA-A2+ Granta 519 cell line, derived from a mantle cell lymphoma, naturally expressing BARF1 (Küppers R, 2003) and LCLs established from HLA-A*0201+ donors.

In vivo experiments were performed using six-to-eight week-old female SCID mice (n=6-10 for all groups), purchased from Charles River Laboratories (Calco, Como, Italy) and housed in our animal facility. Procedures involving animals and their care are in conformity with institutional guidelines that comply with national and international laws and policies (D.L. 116/92 and subsequent implementing circulars). For adoptive immunotherapy experiments, mice were treated by s.c. or i.v. injections with 10×10^6 conventional or BARF1-enriched EBV-CTLs in 0.2 mL RPMI, starting when the tumor volume is about 1 cm^3, and up to a maximum of 3 injections. Untreated mice served as negative controls. Tumor growth was monitored at regular intervals by caliper measurements for a total of 40-45 days. Tumor volume (TV) was calculated using the formula TV (mm^3) = $D \times d^2/2$, where D and d are the longest and shortest diameters, respectively. In selected experiments, monitoring of *in vivo* growth was carried out using tumor cells expressing luciferase after transduction with a lentiviral vector. The animals were regularly analyzed with an instrument specifically dedicated to bioluminescence (Lumina, Caliper/Xenogen). The *in vivo* tumor growth experiments were carried out in collaboration with Dr. Rosato's Group (Padova) and conducted according to the United Kingdom Coordinated Committee on Cancer Research guidelines for the welfare of animals in experimental neoplasia (Moutschen M, 2007), and they were sacrificed when any single tumor mass will exceed 300 mm^3. Kaplan-Meier product-limit method was performed to estimate the survival curves, and comparison of survival between groups will be carried out using the log-rank test. Medians were calculated and reported with their p values, based on a two-sided testing.

PRODUCTION OF BARF1-SPECIFIC MONOCLONAL ANTIBODIES

On the basis of the primary structure and crystallography data (Elliott SL, 2008) a discrete number of BARF1 peptides were identified for immunization experiments. Selected KLH-conjugated BARF1 peptides (10 μg/mouse) in incomplete Freund's adjuvant were used to immunize groups of 6 Balb/c mice with a schedule including 3 inoculations (at day 0, 14 and 21). At day 30, blood samples were collected and sera were analyzed by ELISA on peptide-coated plates and by flow cytometry on cell lines known to express BARF1 mRNA. Spleen cells (10^8) from immunized mice were fused with NS/0 myeloma cells (10^7) with 50% poly-ethylene glycol 1500. After fusion, the cells were suspended in HAT-RPMI1640-15% FCS medium and distributed in 96-well cell culture plates. Positive hybridomas were expanded and large amounts of antibodies were collected for purification through 50% SAS (saturated ammonium sulphate) precipitation and subsequent dialysis. Purified antibodies were then quantified and characterized for the relative isotype.

COMPLEMENT-DEPENDENT CYTOTOXICITY (CDC) ASSAY

Anti-BARF1 mAB at various concentrations (20, 10 and 5 μg/ml) was added to different target cell lines (Granta 519, c.666-1, BL-41 and BL-41 B95.8 cancer cell lines). For the negative control, an unrelated IgM mAB was added to all cell lines. As a source of complement, normal human serum prepared from healthy volunteers was added to the samples. After 2 h at 37°C, the samples were stained with propidium iodide (PI) and then specific CDC activity was determined according to the formula:

$$\% \text{ cytotoxicity} = 100 \times (E-S)/(M-S)$$

where E is the absorbance of experimental well, S is that in the absence of mAb (cells were incubated with medium and complement alone), and M is that of medium and complement in the absence of target cells and antibody.

ANTIDODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY ASSAYS

Some weeks before the test, PBMC effector cells were collected from a healthy blood donor by Ficoll gradient centrifugation and vitally cryo-preserved. PBMCs were thawed and diluted to a final concentration of 2.5×10^6 cells/mL in HBSS-10% FBS and cultured for 2 hours. Meanwhile, the target c.666-1 and Granta 519 cells were collected and re-suspended in HBSS-10% FBS at a final concentration of 10^6 cells/mL. Cells were labeled by adding 6 μM of ca-AM, mixing carefully and incubating for 90 min at 37°C and 5% CO2. Cells were washed thrice with 10 mL HBSS, diluted to 10^6 cells/mL and stained with the 10E5 anti-BARF1 mAB, 60 min on ice. To perform the assay, two different concentrations, 5 and 10 μg, of the mAB were added to each experimental condition and controls included a NO AB condition and an unrelated IgM mAB staining. After staining, the target cells were re-suspended in seeded HBSS-10% FBS and seeded at 3000 cells/well in a V-bottomed 96-well culture cell plate, including the Total Release (TR, target cells with lysis buffer) and the Spontaneous Release (SR, only target cells). Finally effector cells were added in the wells at different E:T ratios (100:1, 50:1, 25:1, 12.5:1 and 6,25:1). Plates were incubated for 4 h at 37°C and 5% CO2, and centrifuged at 700× g for 5 min, then 80μL of each supernatant was transferred into an OptiPlate-96 fb (Perkin-Elmer LAS GmbH). Plates were incubated for 15 min and then fluorescence was measured using the SpectraFluorPlus (Tecan, Austria). Excitation and emission filters were 485 and 535 nm, respectively and gain was set at 70. The percentage of specific release was determined using the following formula:

(experimental release − spontaneous release)/(maximum release – spontaneous release) ×100.

RESULTS

BARF1 mRNA IS DETECTED IN TUMOR BIOPSIES BUT NOT IN NORMAL NASOPHARYNGEAL TISSUES FROM ITALIAN NPC PATIENTS

As a first step, we verified the prevalence of BARF1 expression in a series of NPC from a non-endemic area such as Italy. Using a specific RT-PCR approach, BARF1 mRNA expression was detected in 15/18 (78%) NPC biopsies whereas all 8 normal nasopharyngeal tissues analyzed were negative. Notably, 4 of these normal tissues were obtained from NPC patients whose tumor biopsy was positive for BARF1 mRNA expression. Raji cells and the DAA3 LCL were also negative, whereas BARF1 mRNA was constitutively expressed by Akata and Granta 519 cells and up-regulated in DAA3 cells after EBV lytic cycle induction (Fig 5). These findings indicate that BARF1 is expressed by tumor cells of the majority of Italian NPC.

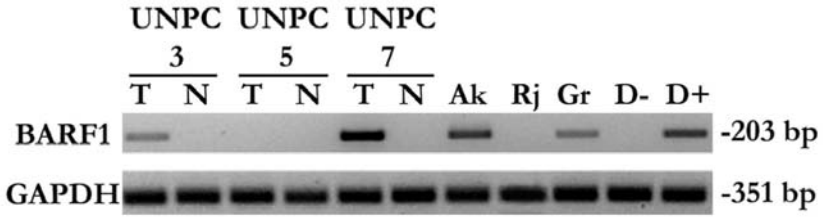

***Figure 5*. RT-PCR analysis of BARF1 mRNA gene expression in tumor (T) and normal (N) tissues of NPC patients.** *About 80% of UNPC tumor biopsies resulted positive to BARF1 mRNA, whereas all samples of normal nasopharyngeal mucosa analyzed were negative. Raji cells and the DAA3 LCL were also negative, whereas BARF1 mRNA was constitutively expressed by Akata and Granta 519 cells and up-regulated in DAA3 cells after EBV lytic cycle induction.*

DETECTION OF BARF1-SPECIFIC T CELL RESPONSES IN HEALTHY DONORS AND NPC PATIENTS

The presence of spontaneous T cell responses to BARF1 protein was investigated in PBMCs from 3 healthy EBV-seropositive donors and 5 UNPC patients by IFNγ-ELISPOT. To this end, autologous monocytes pulsed with the recombinant BARF1 p29 protein were incubated for 48 h with purified CD4+ or CD8+ T cells. Tetanus toxoid was used as a positive control

(median of 47.6 and 50.8 SFC/50 000 for CD4+ or CD8+ T cells, respectively) and gave comparable results in donors and NPC patients. As shown in Figure 6, spontaneous CD4+ and CD8+ T cell responses to BARF1 protein were detected in all these cases, although UNPC patients showed higher numbers of BARF1-specific CD4+ and CD8+ T lymphocytes.

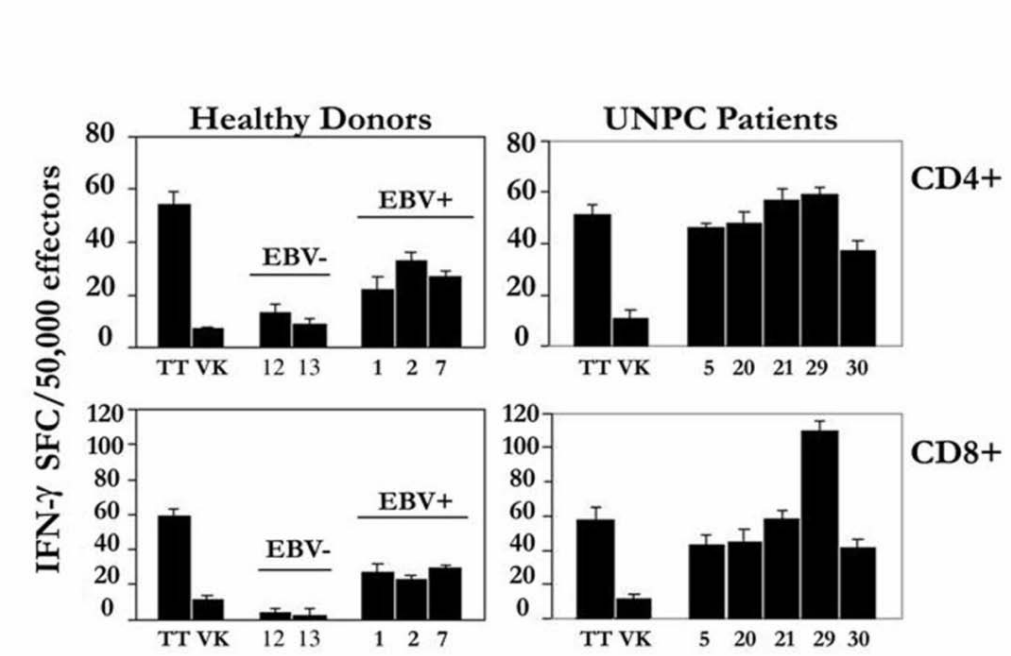

***Figure 6*. Detection of spontaneous CD4+ and CD8+ memory T cell responses to BARF1 protein in EBV-seropositive and EBV-seronegative healthy donors and NPC patients by enumeration of IFN-γ spot forming cells in ELISPOT assay.** *Each bar represents the mean ± SD of quadruplicate wells, after subtraction of background responses given by unpulsed autologous monocytes. Tetanus toxoid (TT) and an unrelated protein (VK) were used as positive and negative controls, respectively, and the results are shown as mean ± SD of the SFC/50 000 effectors detected in the donors and UNPC patients analyzed.*

SELECTION AND BINDING ACTIVITY OF BARF1 PEPTIDES TO HLA-A*0201 MOLECULES

Within the first 60 amino acid sequence of the BARF1 protein, immuno-informatic prediction analysis allowed the selection of ten 9-mer peptides that could potentially bind to HLA-A*0201 molecules (Table 2). Nevertheless, only five of these HLA-A*0201-restricted peptides were available for the study (Table 2), since the high hydrophobicity of the p3-11, p6-14, p7-15, p8-16, and p9-17 peptides prevented their successful synthesis and purification.

aa POSITION	a.a. SEQUENCE	SYFPEITHY score	BIMAS score
2-10	RFIAQLLLL	17	0.03
3-11	FIAQLLLLA	21	7.23
6-14	QLLLLASCV	23	257.34
7-15	LLLLASCVA	18	12.81
8-16	LLLASCVAA	21	31.25
9-17	LLASCVAAG	21	0.29
22-30	AFLGERVTL	20	0.05
23-31	FLGERVTLT	21	323.25
29-37	TLTSYWRRV	20	23.65
49-57	KLGPGEEQV	25	119.28

Table 2. BARF1 peptides and predicted binding to HLA-A*0201

Peptides p2-10, p22-30, p23-31, p29-37, and p49-57 were then tested in the T2 stabilization assay. As shown in Figure 3, the Flu $M1_{58-66}$ gave a FI of 2.3, consistently with its high affinity for HLA-A2. None of the BARF1 peptides analyzed showed high affinity binding to HLA-A2 and only the p49-57 peptide demonstrated a low affinity binding in this assay, giving a FI of about 1.2 (Fig 7). The pan HLA Class I monoclonal antibody W6/32 or the anti HLA-A2 BB7.2 antibody gave similar results (not shown).

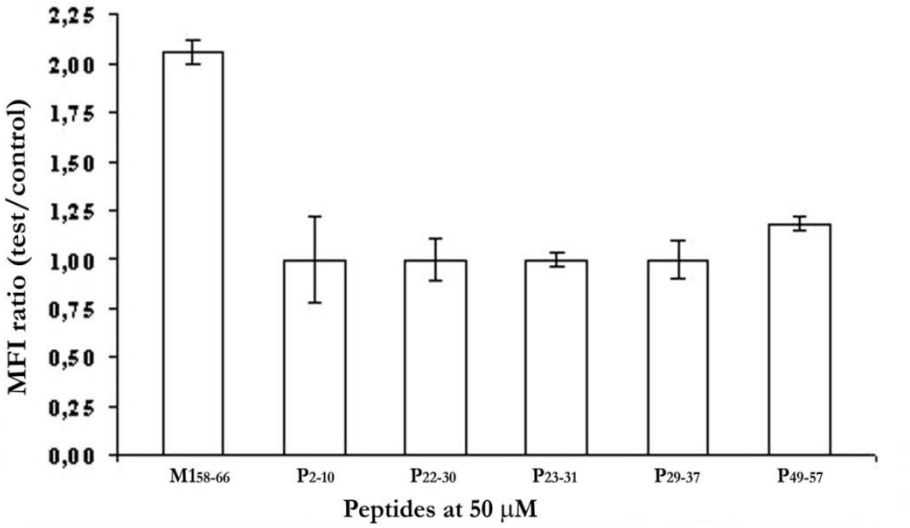

Figure 7. Peptide-MHC class I stabilization assay using T2-A2 cell line. *The fluorescent index (FI) was calculated for each selected peptide, using Flu $M1_{58–66}$ peptide as positive control. Peptides were considered to stabilize HLA molecules with high affinity when the FI was 1.5 and low affinity when the FI was between 1.1 and 1.49. All the assays were repeated a minimum of 3 times, and the results are given as means of replicate experiments.*

SPONTANEOUS T CELL RESPONSES AGAINST BARF1-DERIVED HLA-A*0201 PEPTIDES

Spontaneous CD8+ T cell responses to BARF1-derived peptides (p2-10, p22-30, p23-31, p29-37, and p49-57) were evaluated by IFNγ-ELISPOT in 6 HLA-A*0201+ EBV-positive healthy donors and 9 HLA-A*0201+ NPC patients. Purified CD8+ T lymphocytes were stimulated with BARF1-derived peptides presented by autologous monocytes. Spontaneous CD8+ T cell responses against all 5 BARF1-derived peptides were detected in all donors and NPC patients investigated, although the number of IFNγ-secreting CD8+ T cells was significantly higher in NPC patients for each peptide (Table 3). Notably, the immune responses against the p2-10, p23-31, and p49-57 peptides were slightly dominating in both donors and NPC patients (Table 3). In all three HLA-A*0201-negative donors investigated as a control group, the number of spots induced by the 5 BARF1 peptides was usually at the background level (< 2 SFC/50 000 CD8+ cells) (Table 3).

EBV-HLA-A2 donors	p2–10 (RFI)	p22–30 (AFL)	p23–31 (FLG)	p29–37 (TLT)	p49–57 (KLG)	Flu $M1_{58–66}$ (GIL)
D13	3 ± 1	1 ± 1	2 ± 1	0 ± 1	5 ± 1	63 ± 5
EBV+HLA-A2 donors	p2–10 (RFI)	p22–30 (AFL)	p23–31 (FLG)	p29–37 (TLT)	p49–57 (KLG)	Flu $M1_{58–66}$ (GIL)
D1	8 ± 1	5 ± 1	12 ± 1	5 ± 2	13 ± 1	79 ± 11
D2	10 ± 1	4 ± 2	13 ± 1	4 ± 1	12 ± 1	67 ± 7
D3	24 ± 2	15 ± 1	23 ± 2	6 ± 1	23 ± 1	75 ± 12
D4	32 ± 3	21 ± 1	40 ± 3	23 ± 3	44 ± 3	64 ± 9
D5	21 ± 1	23 ± 2	26 ± 1	13 ± 0	21 ± 1	38 ± 3
D6	57 ± 5	31 ± 1	64 ± 4	26 ± 3	74 ± 8	75 ± 7
Average	25	17	30	13	31	66
EBV+HLA-$A2^-$ donors	p2–10 (RFI)	p22–30 (AFL)	p23–31 (FLG)	p29–37 (TLT)	p49–57 (KLG)	
D9	0 ± 1	2 ± 2	1 ± 1	1 ± 2	1 ± 2	
D10	3 ± 2	1 ± 2	2 ± 2	3 ± 2	1 ± 2	
D11	2 ± 2	3 ± 2	2 ± 2	3 ± 2	3 ± 2	
Average	2	2	2	2	2	

UNPC cases	p2–10 (RFI)	p22–30 (AFL)	p23–31 (FLG)	p29–37 (TLT)	p49–57 (KLG)	Flu $M1_{58–66}$ (GIL)
U 16	93 ± 12	15 ± 2	109 ± 17	76 ± 8	97 ± 12	127 ± 14
U 21	77 ± 11	41 ± 9	95 ± 6	37 ± 3	103 ± 14	121 ± 11
U 22	159 ± 12	83 ± 11	139 ± 12	95 ± 6	143 ± 9	195 ± 15
U 23	61 ± 6	21 ± 2	68 ± 6	12 ± 2	65 ± 6	73 ± 6
U 24	86 ± 4	104 ± 13	107 ± 16	76 ± 5	101 ± 8	153 ± 11
U 25	160 ± 9	157 ± 12	166 ± 9	148 ± 13	200 ± 6	183 ± 17
U 26	23 ± 2	17 ± 2	26 ± 2	9 ± 3	25 ± 3	40 ± 5
U 27	32 ± 3	14 ± 3	36 ± 2	12 ± 3	44 ± 3	49 ± 4
U 28	27 ± 2	13 ± 1	32 ± 7	9 ± 1	34 ± 3	32 ± 2
Average	72	47	78	46	81	97

Table 3: Frequency of CD8+ responders to BARF1- derived peptides in healthy donors and NPC patients. *Spot forming cells/50 000 CD8+ cells (mean ± SD). Purified CD8+ effectors were added to peptide-loaded monocytes at the effector:target ratio of 1:1 and Flu $M1_{58–66}$ peptide was used as positive control. Mean and SD are included. Differences between UNPC patients and donors were statistically significant for each BARF1 peptide ($p < 0.001$).*

INDUCTION OF PEPTIDE-SPECIFIC CTLS AND CYTOTOXIC ACTIVITY OF CTL CULTURES

Peptide-specific CTLs were generated from 2 HLA-A*0201, EBV-seropositive healthy donors by stimulating peripheral blood T lymphocytes with autologous DC loaded with the p2-10, p23-31, or p49-57 peptides. After three weekly re-stimulations, cytotoxic activity of CTL cultures was evaluated using autologous peptide-loaded LCLs as targets in calcein-AM cytotoxicity assays. As shown in Figure 8A, CTL cultures showed HLA-A*0201 peptide-specific killing: T cells only recognized autologous LCLs loaded with the respective BARF1 HLA-A*0201 peptide, whereas no lysis was induced in un-pulsed LCLs. Moreover, pre-incubation with an anti-HLA-A2 antibody markedly inhibited the specific lysis, indicating that the cytotoxic activity of these effectors was HLA-A2-restricted. In the next set of experiments, we evaluated the ability of BARF1 peptide-specific CTLs to lyse tumor cells endogenously expressing the BARF1 protein. To this end, we used as a target the EBV-

carrying Granta 519 cell line expressing both HLA-A*0201 and BARF1. CTLs obtained from two different donors (sharing only the HLA-A*0201 allele with Granta 519 cells) and specific for the p2-10, p23-31, or p49-57 BARF1 peptides were able to efficiently lyse Granta 519 cells in an HLA-A2-restricted fashion, as shown by inhibition of cytotoxicity in the presence of a monoclonal antibody to HLA-A2 (Fig 8B). The extent of specific killing was similar to that induced in peptide-pulsed autologous LCLs (Figure 4B). Again, no lysis of autologous un-pulsed LCLs was observed, confirming the specificity of killing (Fig 8A,B). These data indicate that DC pulsed with BARF1-derived peptides can induce BARF1-specific Class I-restricted CTLs that recognize tumor cells endogenously expressing BARF1 in an antigen-specific and HLA-A-restricted manner *in vitro*. These findings also indicate that the p2-10, p23-31, and p49-57 BARF1 peptides can be processed naturally in cancer cells and can be efficiently presented in the context of HLA-A*0201 on the cell surface of cancer cells to be recognized by CTLs.

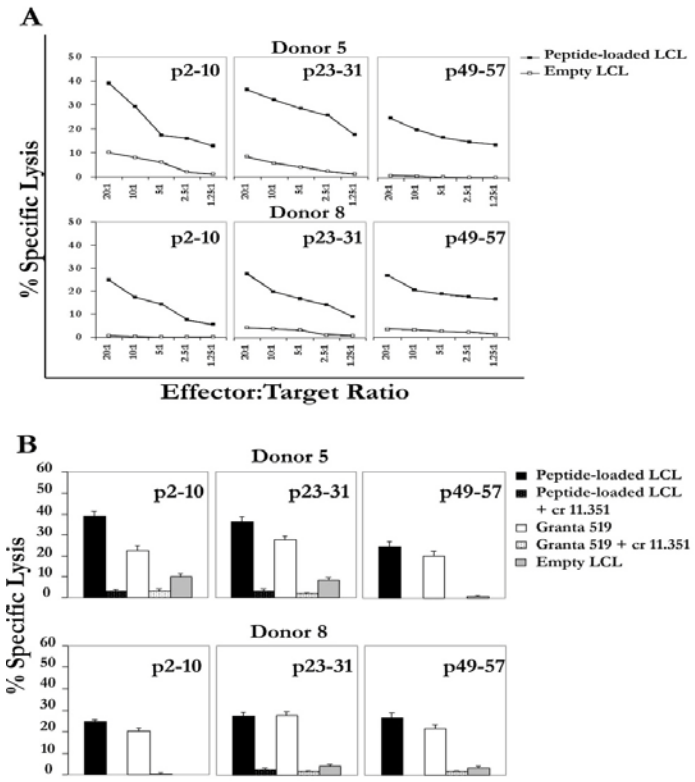

Figure 8: CTL cultures from 2 HLA A*0201 healthy donors were generated against 3 BARF1-selected peptides. (A) *Using peptide-loaded and un-pulsed autologous LCLs as target cells, specific cytotoxic activity was evaluated by standard calcein-AM release assay. All tests were performed in triplicate and at Effector:Target Ratio of 20:1, 10:1, 5:1, 2.5:1 and 1.25:1.* ***(B)*** *Peptide-specific cytotoxic T cells were also able to efficiently lyse tumor cells endogenously expressing the BARF1 protein, in an HLA-A2-restricted fashion, as demonstrated by the incubation of target cells with the anti-HLA-A*0201 cr11.351 mAb. No lysis of autologous un-pulsed LCLs was observed in all performed tests, thus confirming the specificity of killing.*

CONSERVATION OF HLA*A0201 BARF1 EPITOPES AND IDENTIFICATION AND VALIDATION OF NEW CTL EPITOPES

High resolution HLA class I genotyping carried out in 82 Italian NPC patients and 286 bone marrow donors born in the same province showed that the A*0201, B*1801, and B*3501 alleles were significantly under-represented in NPC patients, whereas the A*2601 and B*4101 were over-represented (Fig 9). Notably, these results indicates that the HLA-A*0201 allele is significantly under-represented in these cases as compared to healthy donors matched for age, sex and province of birth (17.7% vs. 32.3%; $p<0.001$) (Fig 9). Considering that the HLA-A*0201 allele also efficiently present a number of other subdominant epitopes of LMP1 and LMP2, the decreased risk (OR: 0.4; 95% CI=0.3-0.7) to develop NPC associated with this allele, therefore, could be related to its ability to promote the induction of efficient CTL responses against EBV+ cells, including those expressing BARF1, such as NPC cells. Moreover, to define more precisely the ability of BARF1 to elicit significant CTL responses in the context of the HLA class I profile of Italian NPC patients, additional BARF1 CTL epitopes were identified and validated, particularly with respect to other common HLA class I alleles that are not under-represented in NPC cases from our area.

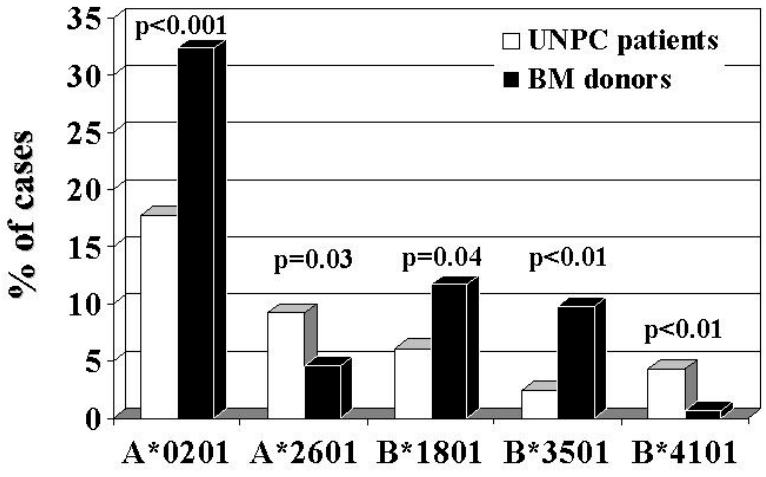

Figure 9. HLA Class I profile of Italian NPC patients as disclosed by high-resolution genotyping. *Only the alleles showing a significantly different distribution are shown.*

Immunoinformatic prediction allowed the identification of 3 HLA*A1101, 2 HLA-A*2402, and 5 HLA-B*5101 candidate BARF1 epitopes (not shown). IFNγ-ELISPOT experiments

demonstrated the presence of spontaneous CD8+ T cell responses to the candidate epitopes in PBMCs of both healthy donors and NPC patients (Fig 10). For some of the investigated epitopes, the extent of responses was higher than that observed in EBV-seropositive healthy donors.

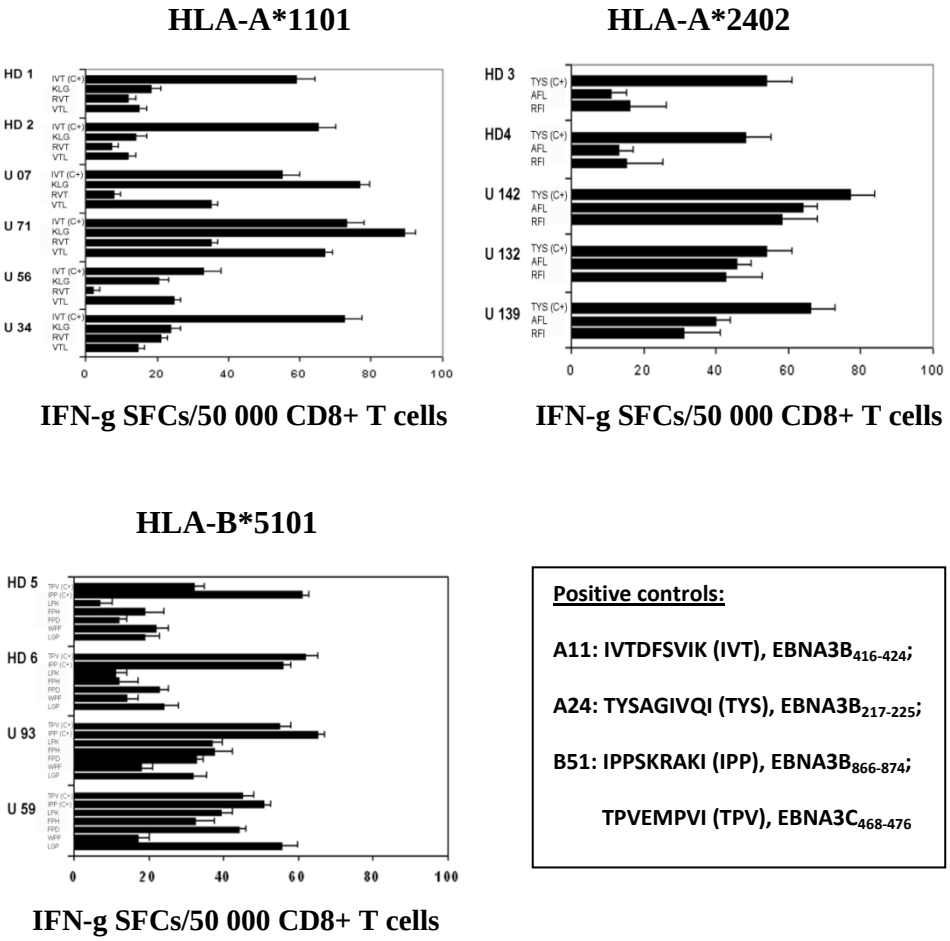

Figure 10: IFNg-ELISPOT assay carried out on purified CD8+ T cells from a preliminary group of EBV-seropositive donors (HD) and NPC patients (U) with 3 HLA*A1101, 2 HLA-A*2402 and 5 HLA-B*5101 predicted BARF1 epitopes. *Each bar represents the mean ± SD of quadruplicate wells, after subtraction of background responses, given by un-pulsed autologous monocytes.*

IN VIVO VALIDATION OF THE THERAPEUTIC EFFICACY OF EBV-CTLS ENRICHED IN BARF1-SPECIFIC EFFECTORS

Considering that the majority of NPC express BARF1 in latently infected tumor cells (Lee ES, 1995; Wei WI, 2005; Küppers R, 2003), as a first priority we used as animal model immunodeficient mice (SCID) grafted with the EBV+ NPC C666 cell line (Hjalgrim H, 2003) spontaneously expressing BARF1. This cell line, however, lacks expression of HLA-A alleles and express only a rare HLA-B allele (B*5801) (Pritzker KP, 1970). Experiments aimed at ectopically and stably expressing the HLA-A*0201 allele in the C666 cell lines are already in progress. BARF1 cDNA has been cloned from a NPC biopsy, verified by sequencing, and inserted in the pLXSN retroviral vector. The *in vivo* growth curves of the selected cell lines grafted s.c. and i.p. in SCID mice have been defined.

DEVELOPMENT AND CHARACTERIZATION OF BARF1-SPECIFIC MONOCLONAL ANTIBODIES

Four potentially immunogenic BARF1 peptides were identified so far (not shown). The first round of immunization experiments carried out with a peptide located at the C-terminus of the BARF1 protein allowed the identification of a first antibody of interest (clone 10E5, IgM isotype). Preliminary data indicate that the 10E5 antibody stain positively EBV+ BARF1+ cells (BL41-B95.8, Granta 519, C666, some LCLs), but not EBV- cells (Ramos, BL41, DG75) in immunofluorescence (data not shown) and flow cytometry (Fig 11). Notably, the antibody did not stain RAJI cells, which are EBV+ but carry a deletion including the BARF1 gene (Papadopoulos EB, 1994) (Figure 11). Moreover, the 10E5 clone mediates significant and CDC (Fig 12) and ADCC (Fig 13) on EBV+ BARF1+ cells but not in EBV- cells.

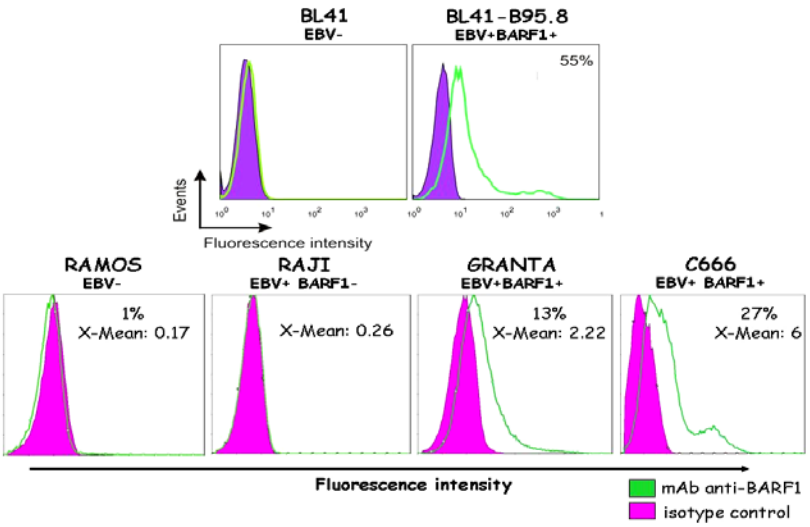

Figure 11. Flow cytometric evaluation of the reactivity of the 10E5 clone in a panel of tumor cell lines with a different status as regards EBV infection and BARF1 expression.

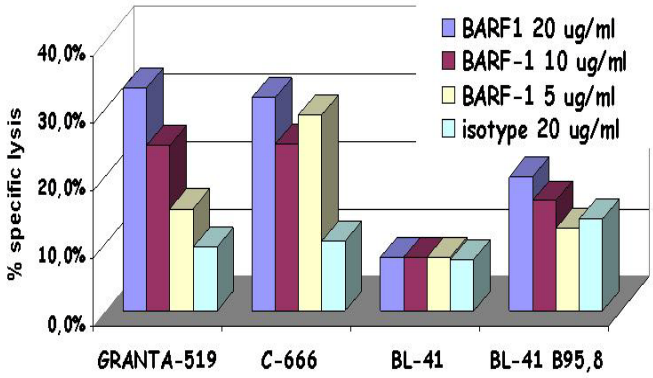

Figure 12. Complement-dependent cytotoxicity mediated by different concentrations of the 10E5 anti-BARF1 mAb on different target cells.

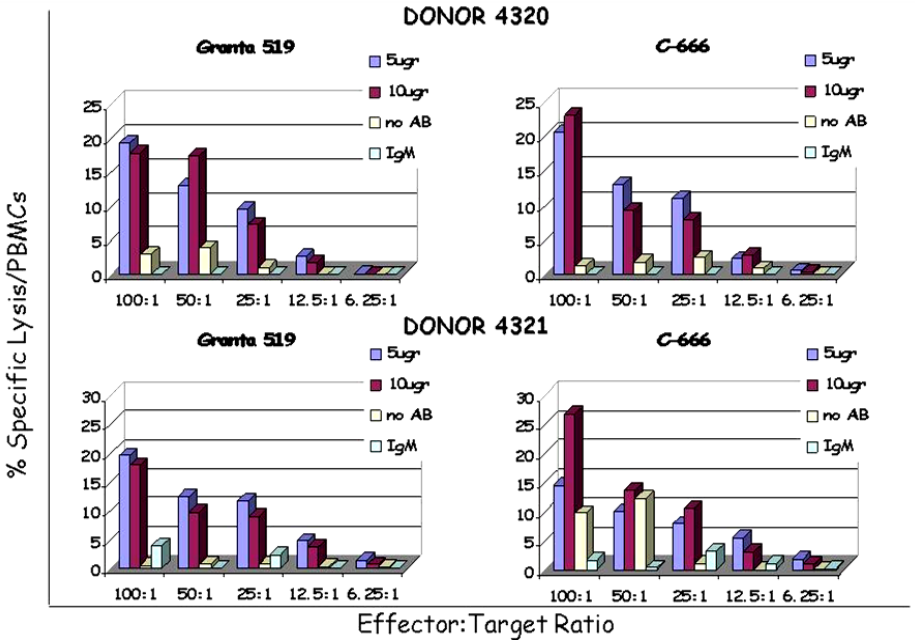

Figure 13. Antibody-dependent cytotoxicity mediated by different concentrations of the 10E5 anti-BARF1 mAb on the Granta 519 and C666 cells*. PBMCs from two unrelated donors have been used at different Effector:Target ratios.*

Moreover, the possible effects on *in vivo* growth of the 10E5 clone were also investigated in a preliminary group of SCID mice grafted with the c.666-1 or the Granta 519 cell lines (Fig 14). The results obtained in this pilot experiment are consistent with a likely *in vivo* antitumor activity of this antibody (Fig 14).

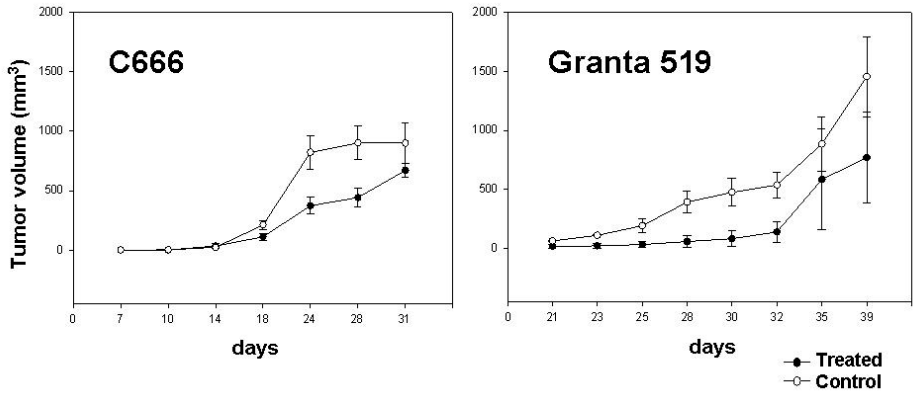

Figure 14. In vivo antitumor activity of the 10E5 clone analyzed in a preliminary group of SCID mice grafted with the c.666-1 or the Granta 519 cell lines. *Animals were treated with 200 μg/mouse/inoculum at days 1, 2, 3, 4 and 5 for the c.666-1 cell line and at 0, 3, 6, 9, and 12 for the Granta 519 cell line.*

DISCUSSION

In the last years, the demonstration of the expression of viral antigens by EBV-associated malignancies has allowed the development of adoptive immunotherapy strategies relying mostly on *in vitro* generation and expansion of virus-specific CTLs, which can be administered to patients for both prophylaxis and treatment (Merlo A, 2008; Leen AM, 2008; Masmoudi A, 2007). Clinical evidence accumulated so far indicates that adoptive therapy with EBV-specific CTLs (EBV-CTLs) is safe, well tolerated and particularly effective in the case of most immunogenic tumors like PTLD (Merlo A, 2008; Leen AM, 2008). However, in latency II EBV-associated malignancies, the more restricted pattern of viral latent antigen expression strongly limits the therapeutic potential of EBV-CTLs obtained by conventional protocols, based on the use of autologous LCLs as source of viral antigens. In fact, the infusion of EBV-targeted autologous CTLs was shown to enhance specific immune responses and to induce objective clinical responses only in a proportion of NPC and HL cases (Lin CL, 2002; Comoli P, 2005; Strathoff KC, 2005; Bollard CM, 2004; Rickinson AB, 1997). This is probably due to the weak immunogenicity of LMP1 and LMP2, which are the most studied proteins in these malignancies, as a consequence of the lack of immunodominant epitopes for CTLs (Khanna R, 1998). Indeed, in healthy EBV-seropositive individuals, the majority of EBV-specific CD8+ T cell responses are directed mainly against epitopes derived from proteins of the EBNA-3 family, which is, however, not expressed in these tumor histotypes (Hislop AD, 2007). Considering that the malignant cells of NPC and HL have functional antigen processing machinery and express HLA and co-stimulatory molecules (Khanna R, 1998; Lee SP, 2000), the demonstration that other viral latent proteins expressed by these neoplastic cells may serve as tumor-associated antigens could provide the rational background to improve the clinical efficacy of adoptive immunotherapy protocols in this setting. On these grounds, BARF1 protein may constitute a potentially useful target to be exploited to improve the efficacy of EBV-based immunotherapy for NPCs since this viral antigen is mainly expressed in EBV-associated carcinomas (Lin X, 2007; Li J, 2007; Ogino T, 2007; Herrmann K, 2003).

Although integrated treatment with radiotherapy and chemotherapy has considerably improved the clinical control of NPC, a significant number of patients relapse, particularly

those with advanced disease at diagnosis (Lau KM, 2007). Moreover, conventional treatments are often accompanied by severe side-effects that may also include secondary malignancies (Khanna R, 1998). Therefore, there is a pressing need to develop new and less toxic therapies able to improve survival of NPC patients. Immunotherapy approaches targeting EBV-encoded viral antigens appear promising in this respect, having shown the ability to induce specific immune responses and objective clinical responses, although only in a fraction of UNPC cases (Dolcetti R, 2003; Carbone A, 2005). In the present study, we have verified that BARF1 is expressed in NPC from a non-endemic region such as Italy. In fact, about 80% of NPC from our series selectively show BARF1 mRNA in tumor biopsies, whereas normal nasopharyngeal tissues are negative. We also provide evidence indicating that BARF1 is naturally immunogenic for T lymphocytes, as shown by the detection of spontaneous CD4+ and CD8+ T cell responses specific for the p29 BARF1 protein in both EBV-seropositive donors and NPC patients. Using bioinformatic prediction tools, we have selected and characterized 5 differentBARF1 CTL epitopes presented by HLA-A*0201, the most frequent HLA class I allele in Caucasians. All these 5 epitopes mapped within the first 60 amino acids of the BARF1 protein, a region that includes the conserved transforming domain. Using the T2-A2 stabilization assay, however, only one (p49-57) of the 5 peptides analyzed was able to bind HLA-A2, although with low affinity. Despite these premises, all 5 BARF1 nonamers available for the study readily elicited specific CD8+ T cell responses in EBV-seropositive HLA-A*0201+ donors, as shown by IFNγ-elispot. In all donors investigated, immune responses against the p2-10, p23-31, and p49-57 peptides tended to be slightly dominating, with a mean frequency of peptide-specific CD8+ T cells corresponding to about half of that observed for the control FLU $M1_{58-66}$ peptide. These findings are consistent with the notion that higher peptide binding affinity for HLA molecules does not necessarily correspond to the functional activity of the responding T lymphocytes (van Beek J, 2006; Hummel M, 1995). The direct detection of strong BARF1-specific responses without pre-stimulation in EBV-seropositive donors suggests the existence of a relatively high frequency of BARF1-specific CD8+ T cell precursors, consistently with what observed for other EBV-encoded proteins, particularly those expressed during EBV lytic replication (Kuppers R, 2003; Kutok JL, 2006). Considering that in normal B cells BARF1 is expressed as an early gene (Herbst H, 1996), the relatively high frequency of BARF1-specific T lymphocytes detected in healthy donors is consistent with a likely relevant role of these effectors in keeping a tight control of EBV replication in the infected host. Notably, the magnitude of CD8+ T cell responses to the whole

BARF1 protein and derived peptides was significantly higher in NPC patients than in healthy donors. This contrasts with what observed for the latent EBV proteins LMP1 and LMP2 whose subdominant epitopes elicited weaker responses in NPC patients as a possible consequence of tumor-related immune suppression (Babcock GJ, 2000; Kapp U, 1999). A possible explanation for this apparent discrepancy may be found in the biological properties of the BARF1 protein, which, unlike LMP1 and LMP2, can be actively secreted by NPC cells. Within tumor microenvironment, therefore, the BARF1 protein may be captured and processed by local DCs or other antigen presenting cells, which may provide a sustained presentation of antigenic BARF1 peptides. This may lead to the increased numbers of BARF1-specific T cells circulating in the blood of NPC patients, although it remains to be elucidated whether these effectors are detectable and functional within NPC lesions.

We also verified whether BARF1 peptides could be used as T cell epitopes able to induce antigen-specific CTLs. This has been accomplished using PBMCs from HLA-A*0201+ donors in an *in vitro* immunization protocol including autologous monocyte-derived DCs pulsed with the BARF1 peptides as antigen presenting cells. Our results show that CTLs specific for the p2-10, p23-31, or p49-57 BARF1 peptides can be easily induced from PBMCs of EBV-seropositive healthy donors. These cultures were not only able to lyse autologous LCLs loaded with the antigenic peptide, but also recognized tumor cells endogenously expressing BARF1 in an antigen-specific and HLA-A2-restricted manner. These findings indicate that the p2-10, p23-31, or p49-57 BARF1 peptides used for CTL induction are also naturally processed and presented as CTL epitopes on BARF1-expressing tumor cells. On these grounds, we further characterized the immunogenic properties of BARF1 and in particular, we verified whether HLA-A*0201 BARF1 epitopes were conserved in EBV isolates from our geographic area. Considering that the HLA-A*0201 allele seems to be under-represented in Italian UNPC patients (our preliminary data), we identified and validated additional BARF1 CTL epitopes presented in the context of other common HLA class I alleles. We selected 3 HLA-A*1101, 2 HLA-A*2402 and 5 HLA-B*5101 restricted epitopes, and demonstrated the presence of higher spontaneous CD8+ T cell responses for some of them in NPC patients, compared to HLA-matched healthy donors. These results gave us a more precise estimate of the immunogenicity of BARF1 in relation to the HLA class I profile of Italian NPC patients and will also allow the development and validation of MHC tetramers to be used in clinical setting to monitor BARF1-specific CD8+ T cell responses at the epitope level. Moreover, to fully exploit the immunologic properties of BARF1, we are now

developing and characterizing some BARF1-specific monoclonal antibodies. Indeed, these reagents could be particularly useful to define more precisely the spectrum of EBV+ tumors that may be treated with strategies targeting BARF1. This could be particularly true for EBV-related lymphoid malignancies, since it is still unclear whether BARF1 is expressed and in which proportion of cells in positive cases. Availability of BARF-1-specific monoclonal antibodies could be useful not only for diagnostic purposes, but it may also be relevant for the development of new therapeutic strategies in this setting, considering the transforming and immunomodulating effects of this EBV-encoded protein.

In conclusion, our results demonstrate that BARF1 provides target epitopes for spontaneous T cell responses in EBV-seropositive healthy donors and, to a greater extent, in NPC patients. These findings, together with the previous observation that BARF1 also elicits strong humoral responses in UNPC patients (Borza CM, 2002), indicate that this protein could be a particularly attractive antigen with immunogenic properties in most NPC patients. Moreover, in future perspective, this study may provide a strong rationale for the clinical application of improved adoptive immunotherapy protocols for the treatment of EBV-associated malignancies, particularly the less immunogenic forms, such as NPC and, possibly, HL. Boosting of BARF1-specific immune responses may contribute to improve the extent and duration of clinical responses in patients with a refractory disease and the subset of cases that still shown a poor prognosis.

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