In vivo targeting and growth inhibition of the A20 murine B-cell lymphoma by an idioype-specific peptide binder

Camillo Palmieri¹, Cristina Falcone¹, Enrico Iaccino¹², Franca Maria Tuccillo³, Marco Gaspari¹, Francesca Trimboli², Annamaria De Laurentiis¹, Laura Luberto¹, Marilena Pontoriero¹, Antonio Pisano¹, Eleonora Vecchio¹, Olga Fierro⁴, Maria Rosaria Panico⁵, Michele Larobina⁵, Sara Gargiulo⁵, Nicola Costa¹, Fabrizio Dal Piaz⁶, Marco Schiavone⁷, Claudio Arra³, Aldo Giudice³, Giuseppe Palma³, Antonio Barbieri³, Ileana Quinto¹, Giuseppe Scala¹*

¹Department of Experimental and Clinical Medicine, University of Catanzaro “Magna Graecia”, 88100 Catanzaro, Italy; ²Italsistemi Biotechnology Institute, Crotone, Italy; ³Department of Experimental Oncology, National Cancer Institute - INT, Foundation G. Pascale, Naples; ⁴Institute of Food Sciences-National Research Council, Avellino, Italy. ⁵Institute of Biostructure and Bioimaging, National Research Council, Naples, Italy; ⁶Department of Pharmaceutical Sciences, University of Salerno, Salerno, Italy; ⁷Department of Biochemistry and Medical Biotechnology, University Medical School of Naples “Federico II”, 80131 Naples, Italy.

*Correspondence should be addressed to Giuseppe Scala, ¹Department of Experimental and Clinical Medicine, University of Catanzaro “Magna Graecia”, 88100 Catanzaro, Italy, email scala@unicz.it; tel. +39961-3694093; Fax +39961-3694090
ABSTRACT

B-cell lymphoma is a clonal expansion of neoplastic cells that may result in fatal outcomes. Here, we report the in vivo targeting and growth inhibition of the aggressive A20 murine B-cell lymphoma by the idiotype-specific peptide pA20-36. pA20-36 was selected from random peptide libraries and bound specifically to the BCR of A20 cells in mice engrafted with A20 lymphoma, as shown by histology and MicroPET analysis. BCR cross-linking of A20 cells with pA20-36 resulted in massive apoptosis of targeted tumor cells and in an increased survival of the diseased animals without any detectable evidence of toxicity. The pA20-36 treatment reverted the immune suppression of the tumor microenvironment as shown by the reduced expression of VEGF, Th2/Treg IL-10 and TGFβ cytokines together with a lower number of CD11b+Gr-1+ inhibitor myeloid-derived suppressor cells and Foxp3+ CD4+ regulatory T (Treg) cells. Further, the pA20-36 treatment was associated with an increased number of tumor-infiltrating, activated CD8+ T cells that exerted a tumor-specific cytolytic activity. These findings show that a short peptide that binds specifically to the CDR regions of the A20 B-cell receptor allows the in vivo detection of neoplastic cells together with a significant inhibition of the tumor growth in vivo.
INTRODUCTION

Therapeutic advance in the clinical treatment of B-cell lymphoma has taken advantage from the development of targeted therapy based on monoclonal antibodies (MAbs), including CD20, CD22, and CD52, which target cell surface receptors of neoplastic cells\(^1\). Therapeutic MAbs have been shown to be effective either alone, or in combination with chemotherapeutics, and as vehicle to deliver radionuclides or toxins to neoplastic cells. However, the effectiveness of therapeutic MAbs is limited by their extended half-life and toxicity as a consequence of non-specific binding to the reticule-endothelium and to unaffected organs, including the bone marrow\(^2\). This drawback is critical in the case of antibodies used as a vehicle to deliver radionuclides, cytotoxic drugs, or toxins to tumour sites. Hence, although the introduction of therapeutic MAbs has broadened the treatment options for subjects with B-cell lymphoma, a substantial number of patients experience a clinical relapse as a consequence of a lack or poor response to the treatments. This grim picture calls for new strategies to improve the \textit{in vivo} detection and treatment of neoplastic B cells.

As compared to antibodies, peptides are attractive alternatives for cell targeting and \textit{in vivo} cancer imaging and therapy. Indeed, peptides, which are considerably smaller than antibodies and antibody fragments, do not bind to the reticule-endothelium and do not elicit a significant immune response upon repeated inoculation\(^3\). Peptides with blocked \textit{N}- or \textit{C}-terminus may acquire a cyclic, constrained conformation, and may include unnatural and D-amino acids that allow an extended half-life \textit{in vivo}. Also, peptides are endowed with several functions, including multi-valence for target moieties and cargo delivery, including radionuclides, cytotoxic drugs and toxins that target cell surface receptors of cancer cells. Examples of tumor-specific peptides include somatostatin\(^4\) and bombesin/gastrin-releasing peptide (BN/GRP)\(^5\), which exhibit high affinities for the cognate cell-surface receptors. Peptides also facilitate the transport of cytotoxic compounds and stretches of nucleic acid into specific tumor tissues\(^6\).

Random Peptide Libraries (RPLs) allow the selection of therapeutic peptides for tumor cell surface receptors\(^7\). RPLs express a large collection of peptide sequences (10\(^8\) or more) that mimic...
both linear and conformational epitopes of folded protein domains, which are displayed in multiple copies fused to phage coat proteins. In addition, the linear or conformational epitopes displayed on phages can be translated in short cyclic peptides with little or no loss of binding specificity.

The hypervariable regions (idiotypes) of the surface immunoglobulin (Ig) B-cell receptor (Ig-BCR) expressed by lymphoma cells result from the rearrangement of the Ig genes and are unique for a given clonal B cell population. In this setting, the idiotypic determinants of the Ig-BCR expressed by lymphoma cells function as specific tumor antigens that may be exploited for cell-specific targeted therapy. Previous studies using RPLs identified peptides (Id-peptide) that exhibited anti-tumor activity against human and murine B-lymphoma by triggering apoptosis of target cells in vitro when administered as dimers or tetramers.

In this study, we evaluated the tumor targeting properties of an Id-peptide, which was selected from RPLs by using as a bait the Ig-BCR of the highly aggressive murine A20 B-lymphoma. The RPL-selected Id-peptide specifically bound the A20 cells both in cell cultures and in A20-engrafted syngenic immune competent mice, as shown by FACS, confocal microscopy and positron emission tomography (PET). Cross-linking of the A20 BCR with Id-peptide resulted in the massive apoptosis of targeted cells in vitro by a caspase-dependent mechanism. Consistently, inoculation of the Id-peptide in A20-engrafted mice caused apoptosis of tumor cells and resulted in tumor growth inhibition and extended survival of diseased animals. Since apoptotic tumor cells are source of antigens for the immune system, we also performed an extensive analysis of tumor microenvironment to evaluate the impact of Id-peptide treatment on tumor immunity. Indeed, the Id-treatment reverted the immunosuppressive characteristics of the tumor microenvironment and stimulated a tumor-specific CD8+ T cell activity that contributed to the peptide-driven anti-tumor activity.
MATERIALS AND METHODS

Cell lines, immunoglobulin purification and peptides.

The A20 and 5T33MM cell line growth conditions are reported in Supplemental Data. The A20-Ig was purified from the culture supernatants by using the Mab Trap™ antibody purification Kit (GE Healthcare). All peptides were purchased from Caslo laboratory ApS, Lyngby, Denmark. The amino acid sequence of the control scrambled peptide (pCNT) was: DQEWCKTISFEPCLEN.

Generation and screening of RPLs. We generated two RPLs consisting of nona-peptides (f88-C7C) and dodeca-peptides (f88-12) displayed on the N-terminus of pVIII major coat protein of filamentous bacteriophage f88-tet, as previously described9,18. A third f88-Cys6 RPL was a kind gift of G.P. Smith18. Screening was performed by using A20-Ig purified from culture supernatants. Details for constructions and screening of RPLs, as well as ELISA procedures are reported in Supplemental Data.

Peptide binding.

Binding of peptides to purified immunoglobulins, mice sera or cells, and competitive peptide binding inhibition were evaluated by ELISA and described in Supplemental Data.

Cell viability, cell cycle and apoptosis. A20 cells (2 x 10^7/ml) were incubated with biotinylated peptides (20 μg/ml). For peptide tetramerization, streptavidin (100 μg/ml) was added to the cell cultures. Cell viability was determined by trypan blue dye exclusion; apoptosis was measured by Annexin V-FITC staining (Apoptosis Kit, Miltenyi Biotec) and cell cycle was analyzed by using CycleTest Plus DNA Reagent Kit (BD Biosciences); Caspases activities were measured by luminometric assay (Promega Corporation).

Histological analysis. Detailed procedure for histological evaluation of metastasis, apoptosis and
intra-tumoral microvessel density, are reported in Supplemental Data online.

**In vivo studies.** The Institutional Animal Care and Use Committee of the University “Magna Græcia” Catanzaro approved the animal protocols, according to the guidelines of the National Institute of Health, Italy. Detailed procedures for *in vivo* toxicity, micro-PET imaging and tumor growth inhibition analysis are reported in Supplemental Data online.

**Analysis of tumour infiltrating leukocytes.** Analysis of tumour infiltrating leukocytes was performed by flow cytometry. Experimental details are reported in Supplemental Data online.

**T cell functional assays.** CD8+ T cells were isolated from tumour infiltrating lymphocytes by using an indirect magnetic labelling system (MACS, Miltenyi Biotec), according to the manufacturer's instruction. Purified CD8+ T cells were labelled with CFSE (Molecular Probes) and re-suspended in RPMI 1640 supplemented with 5% FCS. Purified CD8+ T cells (5 x 10^4 cells/well) were mixed with cultured A20 or 5T33MM cells (2 x 10^5 cells/well) and seeded into a 96-well plate. After 1 week of incubation, CFSE-labelled cells were analyzed by flow cytometry. To assess the tumour-specific cytolytic activity of the tumour infiltrating CD8+ cells, a time-resolved fluorometric assay (DELFIA, Perkin Elmer) was performed according to the manufacturer's instruction.
RESULTS

Identification of peptide ligands of the A20 monoclonal immunoglobulin.

To identify peptide ligands of surface IgG (sA20-Ig) of A20 B-lymphoma cell line we screened three f88 phage-displayed RPLs using as bait purified A20-Ig, as previously reported. After three rounds of panning with A20-Ig, 22 phage clones, which tested positive for A20-Ig binding in ELISA, were selected (Table 1); among these, A20-1, A20-6 and A20-36 showed the highest binding affinities to A20-Ig (Fig. 1A, *left panel*). Synthetic peptides pA20-1, pA20-6 and pA20-36 corresponding to the insert peptides of A20-1, A20-6 and A20-36 phage clones, respectively, showed a concentration-dependent binding to A20-Ig (Fig. 1A, *middle panel*), while testing negative for mouse polyclonal Igs (Fig. 1A, *right panel*).

Competition of pA20-1, pA20-6 and pA20-36 for the same binding site of A20-Ig BCR was assessed by an inhibition assay (Fig S1C). In this assay, the binding of N-biotinylated pA20-36 to A20-Ig was inhibited by increasing amounts of either unbiotinylated pA20-1, or pA20-6, albeit to a less extent as compared to the inhibition of unbiotinylated pA20-36, indicating that pA20-36, pA20-1 and pA20-6 bound to the same binding site with different affinities (Fig S1C). The scrambled pCNT did not inhibit the binding of pA20-36 to A20-Ig (Fig. S1C).

Flow cytometry analysis using FITC-conjugated peptides indicated that pA20-1, pA20-6 and pA20-36 bound specifically to A20 cells in a dose-dependent manner (Fig. 1B and Fig. S1A and B). Due to the high affinity binding to A20 cells, the pA20-36 peptide was selected for further evaluation.

The molecular form of pA20-36 was assessed by mass spectrometry. MALDI-TOF analysis revealed that the peptide was present in its monomeric form, bearing an intramolecular disulfide bridge (Fig S2).

The binding affinity value of the A20-IgG:pA20-36 pair was 410 nM Kd as determined by surface plasmon resonance (SPR-Biacore) (Fig. 1C). Further, the pA20-36 peptide and sA20-IgG co-localized at the A20 B cell surface by confocal microscopy (Fig. 1D).
**Functional outcome of pA20-36 peptide binding to sA20 Ig.**

Next, we tested whether the pA20-36 binding to sA20-Ig could induce calcium mobilization upon BCR signaling. The pA20-36 induced calcium fluxes in A20 cells, which were significantly increased by a tetrameric form of streptavidin-linked pA20-36 (Fig. S3A); scrambled pCNT peptide was ineffective.

As Ig-BCR engagement results in antigen uptake by BCR-mediated endocytosis\(^20\), we tested whether the binding of pA20-36 peptide to A20 cells could result in peptide internalization. The incubation of A20 cells with pA20-36 peptide resulted in peptide internalization upon 1 h incubation, as shown by confocal microscopy (Fig. S3B). The kinetics of BCR-mediated internalization were similar in the case of anti-mouse IgG F(ab’2) and pA20-36 peptide (Fig. S3C). The degree of internalization was measured as the percentage of cells no longer expressing surface peptides, assuming \(T_{50}\) as the time at which we observed 50% of BCR-IgG internalization. Despite a slight delay of pA20-36 internalization as compared to the F(ab’2) fragments (\(T_{50} \sim 50’\) versus \(T_{50} \sim 30’\)), similar kinetics and a complete internalization at three hours post-stimulation were observed in the case of the F(ab’2) fragments and the pA20-36 peptide (Fig. S3C). As previously reported\(^20\), the whole antibody was not internalized (Fig. S3C).

Since crosslinking of the Ig-BCR results in growth-inhibiting signals that induced cell cycle arrest and apoptosis in B-cell lymphoma cells\(^21\), we tested whether the pA20-36 binding to A20 cells could affect cell division and apoptosis. To cross-link surface Ig-BCR, we used biotinylated peptides bound to tetravalent streptavidin\(^13,14\). Cell viability was strongly impaired by *in vitro* incubation of A20 cells with either streptavidin-linked pA20-36, or anti-IgG F(ab’)2 antibodies up to 72 h, while it was not significantly affected by monomeric pA20-36, pCNT or streptavidin-linked pCNT peptide (Fig. 2A). The cell cycle analysis showed that the monomeric pA20-36 treatment reduced the G2 population after 24-48 h, and significantly increased the sub-G1 cell population (apoptotic cells) (Fig. 2B, and Fig. S4A). Moreover, the tetrameric pA20-36 treatment resulted either in a more consistent reduction of G2 population after 24-48 h, and in 4-5 fold increase of the
sub-G1 cell population at 48 h, as compared to the monomeric pA20-36. Consistently, the monomeric pA20-36 treatment resulted in a slight but significant increase of apoptosis, as assessed by Annexin-V binding, while it did not occur upon pCNT peptide treatment (Fig. 2C), while the tetrameric pA20-36 treatment resulted in 4-5 fold increase of apoptotic cells compared to the monomeric pA20-36. Both monomeric and tetrameric pA20-36 treatments did not affect the growth of the 5T33MM cells (Fig. S4B), a surface IgG-positive B cell line unable to bind to pA20-36 peptide (Fig. S1B), thus ruling out that the apoptotic death triggered by pA20-36 was due to a non-specific toxic effect. The activity of the initiator caspase-9 activity was induced by pA20-36 at 24 h treatment followed by the activation of effector caspase-3/7 at 48 h, whereas caspase-8 activity was unaffected; these caspases were not activated by the scrambled pCNT peptide (Fig. 2D). Altogether, these results underscored that A20 tumor cells treated with pA20-36 peptide were driven toward a selective arrest of cell cycle at the G2 phase and a caspase-dependent apoptosis.

**A20 B-lymphoma targeting in vivo by pA20-36.**

To test the in vivo targeting ability of pA20-36, we used the A20 syngenic model of murine lymphoma. BALB/c mice were subcutaneously (s.c.) injected with A20 cells, and at day 12 (tumor volume 63±39 mm$^3$) single cell suspensions from tumor at the site of injection were analyzed for binding to the FITC-conjugated pA20-36 or control peptides; 80% of B220$^+$ B cells from tumor were positive for pA20-36-FITC binding, whereas only 3.2% of them tested positive for the pCNT-FITC binding (Fig. 3A, upper panels). To investigate the ability of pA20-36-FITC to detect metastatic A20 B cells, single cell suspensions from spleen of A20 tumor-bearing mice were analyzed; the pA20-36-FITC peptide specifically bound to 3.6% of B220$^+$ spleen cells as compared to 0.6% of B220$^+$ spleen cells bound by the control peptide (Fig. 3A, middle panels); as expected, the pA20-36-FITC and pCNT-FITC peptides bound to a 0.1-0.2% of B220$^+$ spleen cells from tumor-free mice (Fig. 3A, lower panels). Confocal microscopy confirmed the property of pA20-36-FITC to detect niches of metastatic cells in spleen of tumor bearing mice (Fig. 3B and Fig. S5). The
tumor targeting of pA20-36 peptide was also assessed by micro-PET using $[^{18}\text{F}]$-radiolabeled peptides, as a tracer. Since A20 tumor cells release the A20-Ig paraprotein, we first determined the concentration of serum A20-Ig, which might bind to pA20-36 peptide and prevent its binding to A20 cells. To this end, we developed a sensitive ELISA, which detected serum A20-Ig concentrations up to 0.1 ng/ml (Fig. S6A). In A20 tumor engrafted mice, serum A20-Ig levels ranged between 8-17 ng/ml at day 7 (tumors undetectable by palpation; n=3), and 3.5-15.1 μg/ml at day 12 (tumor volume 63±39 mm$^3$ n=3) (Fig. S6B). The micro-PET analysis was performed in mice harboring A20 B lymphoma engrafted mice at day 12 post-engraftment, when tumor volume ranged within 63±39 mm$^3$, using 10 μg of $[^{18}\text{F}]$-Id-peptides corresponding to 100- 20 molar excess over the A20-IgG serum concentration. MicroPET analysis showed a clear tumor uptake of $[^{18}\text{F}]$–pA20-36 in the BALB/c mice bearing s.c. A20 tumors (Fig. 3C left panels, video1.avi). All time points were monitored from 10 to 60 minutes; mice bearing s.c. 5T33MM tumors tested negative for $[^{18}\text{F}]$–peptides (Fig. 3C, right panels), indicating a specific accumulation of $[^{18}\text{F}]$–pA20-36 in the A20 tumor. Conversely, the $[^{18}\text{F}]$–pCNT did not show tumor uptake in the BALB/c mice bearing s.c. A20 tumors (Fig. 3C, middle panels video2.avi). This analysis also showed a non specific accumulation of all radiolabeled peptides in the kidney, urinary bladder and gastrointestinal tract, which is indicative for a clearance of radiolabeled peptides through the urinary bladder and gastrointestinal tracts.

**pA20-36 inhibits the growth of A20 B-lymphoma in engrafted mice.**

In order to evaluate the in vivo toxicity of Id-peptides, groups of tumor-free BALB/c mice were daily inoculated i.p. with either pA20-36-, or pCNT-peptide (200 mg kg$^{-1}$d$^{-1}$) for two weeks. Both vehicle- or peptides-treated mice showed normal levels of the inflammatory cytokine IL1-β, and the hepatotoxicity marker GSH and LDH (Table 2).

Next, we analyzed the outcome of pA20-36 inoculation on tumor growth in mice engrafted with A20 B-lymphoma. Following s.c. injection of a large tumor load (5x10$^6$ tumor cells) a
detectable tumor mass appeared at day 10 from engraftment. Daily administration of pA20-36 (20 mg kg\(^{-1}\) d\(^{-1}\)) starting 24 h post-engraftment delayed the appearance of tumor masses up to 25 days (Fig. 4A, left panel), while it did not affect the s.c. growth of 5T33MM control tumor (Fig. 4A, right panel); differently, the administration of pCNT did not affect the A20 tumor growth (Fig. 4A, left panel). Injection of a reduced tumor load (5 x 10\(^5\) tumor cells) resulted in increased tumor growth inhibition upon treatment with the pA20-36 peptide (Fig. 4A, middle panel). A similar outcome was observed in the case of the pA20-36 treatment starting at 2 weeks post-engraftment (Fig. S7). Mice survival was significantly affected by the pA20-36 treatment. In fact, mice engrafted with a large (5x10\(^6\) tumor cells) or small (5x10\(^5\) tumor cells) tumor load, and treated with pA20-36, showed 64 % or 70% survival rates, respectively, at day 40, as compared to the death of control groups treated with the pCNT peptide or left untreated (Fig. 4B).

Treatment of B-cell lymphoma with anti-idiotype antibodies can give arise to idiotype variants\(^2\). Thus, we evaluated whether the residual A20 B lymphocytes in engrafted mice upon pA20-36 treatment could result in the emergence of A20 idiotype variants. By flow cytometry, B220\(^+/\)pA20-36\(^+\) B cells were detected at a similar frequency after 24 days of tumor treatment with pA20-36 or pCNT peptides (Fig. S8A), which indicated that the antigenicity of B220\(^+\) tumor cells was not affected by pA20-36 peptide treatment. Further, gene sequence analysis of the IgH variable regions (CDRs) of A20 tumor cells from both pCNT- and pA20-36-treated mice showed a unique nucleotide sequence of both the framework (FW) and the complementary determinant regions (CDR) (Fig. S8B); the nucleotide sequence overlapped with the one expressed by the original A20 cell clone\(^2\), indicating that the minimal residual disease observed in pA20-36-treated mice did not result from mutant, idiotypic escape of tumor IgG at day 24 post-treatment.

To address the mechanism of tumor growth inhibition by pA20-36, we analyzed the levels of apoptosis in pA20-36-treated tumor tissues. Flow cytometry of single tumor cell suspensions showed a statistically significant, higher number of Annexin V-positive cells in pA20-36- treated mice as compared to pCNT-treated mice at day 23 (Fig. 4C). Accordingly, the histology of fresh
tumor masses confirmed the presence of large areas of apoptotic cells in pA20-36-treated tumors, which were undetected in tumor treated with pCNT (Fig. 4D). To analyze the effect of pA20-36 on apoptosis in vivo, syngenic BALB/c mice were i.p. injected with CFSE-labeled A20 tumor cells. At day three post-inoculation, mice were i.p. injected with pA20-36 or control peptide (100 μg/100 μl PBS), and 6 hr later single cell suspensions were isolated from lymph nodes. We observed a significant increase of sub-G1 DNA content in mice inoculated with pA20-36 as compared to control mice (Fig. 4E left). Control CFSE-labeled 5T33MM cells were not significantly affected by the pA20-36 peptide treatment (Fig. 4E right), indicating that pA20-36 specifically induced apoptosis of A20 B cells in vivo.

Analysis of tumor microenvironment.

Next, we addressed the impact of the Id-peptide treatment on anti-tumor immunity within the tumor microenvironment of the A20 B lymphoma-engrafted mice. First, we evaluated the expression levels of cytokines in tumor tissues by real time PCR of mRNAs. The pA20-36 treatment resulted in a reduced expression of immunosuppressive cytokines genes (Tgf-β and Il-10), and pro-angiogenic genes (VEGF, Thromboxane synthase) in the B-cell lymphoma-engrafted mice, while no significant effect was observed in the case of Ifn-γ (Fig. 5A). The immunosuppressive cytokines IL-10 and TGF-β promote the development and activity of Foxp3+ CD4+ regulatory T (Treg) cells; consistently with the reduced expression of IL-10 and TGF-β, Foxp3 expression was reduced in pA20-36-treated mice (Fig. 5A), indicating that the pA20-36-treatment could affect the number of tumor infiltrating Treg cells.

The reduced expression of pro-angiogenic cytokines in tumor tissues from pA20-36-treated mice was investigated by assessing the outcome of pA20-36 treatment on tumor angiogenesis. Immune fluorescence analysis using anti-CD31 MAb as marker of endothelial cells revealed a 25% reduction of the micro-vessel density in tumor tissues of pA20-36-treated mice, as compared to
pCNT mice (Fig. 5B and C). Altogether, these results indicated that the decreased expression of pro-angiogenic cytokines in pA20-36-treated tumors resulted in reduced tumor angiogenesis.

Next, we analyzed the tumor-infiltrating immune cell populations. The pA20-36 treatment did not significantly affect the absolute number of both CD11c+ dendritic cells and CD11b+ macrophage cells in tumor tissues, while we observed a significant decrease in CD11b+ Gr-1+ cells, which includes the myeloid derived suppressor cells (MDSC) that are involved in immune suppression26 (Fig. 6A). The pA20-36 treatment also decreased the number of CD4+, CD8+ T and Treg cells (Fig. 6A).

Further, we analyzed the activation status of tumor infiltrating immune cells. No significant difference was observed in the expression of activation markers (CD80, CD86, MHC-II, PD-L1 and CD40) in CD11c+ dendritic and CD11b+ macrophage tumor-infiltrating cells between pCNT- and pA20-36-treated mice (Fig. 6B). Remarkably, B220+ pA20-36+ cells from pA20-36-treated tumors showed an increased expression of the activation markers CD80, CD86, MHC II, PD-L1 and CD40, thus pointing to an increased antigen-presenting capability of tumor B cells in vivo upon pA20-36 treatment (Fig. 6B). The activation status of A20 tumor cells occurred selectively in the context of the tumor microenvironment, since it was not detected in cultured A20 cells stimulated with pA20-36 (Fig. S9).

Tumor infiltrating CD8+ T cells expressed lower levels of the negative co-stimulatory molecules CTLA4 and PD-1 in pA20-36-treated mice as compared to pCNT-treated mice; CD62L, a marker of naïve and central memory T cells 27, was also down-regulated in pA20-36-treated mice (Fig. 6C, upper panels). Differently, tumor infiltrating CD4+ T cells showed similar expression levels of CTLA4, PD-1 and CD62L in pA20-36- and pCNT-treated mice (Fig. 6C, lower panels). These findings indicated that tumor tissues from pA20-36-treated mice were infiltrated by activated CD8+ T cells. Consistently, CD8+ granzyme B+ T cells were selectively detected in tumor tissues of pA20-36-treated mice (Fig. 6D and Fig. 7A).
CD8+ T lymphocytes contributed to the antitumor activity of the Id-peptide.

As a consistent number of CD8+ T lymphocytes infiltrated the A20 B lymphoma in pA20-36-treated mice, we addressed the role of T cells in the observed tumor inhibition. Increased intracellular production of IFN-γ and granzyme B together with a decreased production of IL-2 was observed in CD8+ T lymphocytes isolated from pA20-36-treated mice, as compared to pCNT-treated mice (Fig. 7A), which was indicative of cell differentiation toward activated T cells. Further, intra-tumor CD8+ T cells were stained with CFSE as a marker of cell proliferation, incubated with A20 cells, and analyzed by flow cytometry. After 1 week of CFSE staining, proliferating CD8+ T lymphocytes were > 30% in the case of pA20-36-treated tumors as compared to 4.0 % of pCNT-treated tumors (Fig. 7B). CD8+ T cell proliferation was specifically induced by A20 B cells since incubation with 5T33MM was unable to stimulate CD8+ T lymphocytes (Fig. 7B). These results show that the high proliferation rate was restricted to tumor infiltrating CD8+ T cells derived from pA20-36-treated mice in the presence of A20 tumor cells. As the A20 stimulating cells used in the proliferation assay had never encountered the pA20-36 peptide, these results underscored that CD8+ T cells from the pA20-36-treated mice recognized tumor epitopes of A20 cells. Consistently, formaldehyde-fixed A20 B cells stimulated a CD8+ T cell proliferation albeit in a smaller cell percentage, as a likely outcome of a reduced antigenicity of tumor cell membrane proteins caused by the formaldehyde treatment (Fig. 7B).

Next, we investigated the cytolytic activity of tumor infiltrating CD8+ T cells. The CD8+ T cells isolated from pA20-36-treated mice showed a dose-dependent killing activity against cultured A20 cells, whereas they did not significantly react against 5T33MM control cell line (Fig. 7C). Conversely, CD8+ T cells from pCNT-treated mice showed a weak cytolytic activity against both A20 and 5T33MM cells, which indicated the level of non-specific bystander effect (Fig. 7C). Collectively, these results show that tumor infiltrating CD8+ T cells from mice treated with pA20-36 elicited an effective immune response toward A20 tumor antigens in vitro.
We next evaluated the *in vivo* contribution of CD8$^+$ T cells to anti-tumor activity by antibody-mediated depletion of CD8$^+$ T cells. To this end, mice depleted of CD8$^+$ T lymphocytes were s.c. injected with A20 B lymphoma and analyzed for tumor growth as compared to undepleted animals. Then, mice were randomly assigned to receive either pA20-36 treatment, or vehicle, beginning at day 1 of post-tumor engraftment. In the absence of pA20-36 treatment, depletion of CD8$^+$ T cells affected the unchecked development of A20 tumors, as shown by the significant increase in tumor growth in CD8$^+$ depleted mice, as compared with un-depleted mice at day 24 (Fig. 7D, *left panel*). In pA20-36-treated groups of mice, we observed a further significant increase of tumor growth in CD8$^+$ T cell-depleted mice, as compared to un-depleted mice (Fig. 7D, *right panel*), pointing to a relevant contribution of CD8$^+$ T lymphocytes to the antitumor activity of the pA20-36 peptide.
DISCUSSION

Tumorigenic B cell lymphomas are sensitive to anticancer treatments, including conventional chemotherapy, radiation therapy and corticosteroids\(^{28}\). Nevertheless, the disease is associated with incomplete response to clinical treatments that results in a minimal residual disease where a few neoplastic cells undetected \textit{in vivo} replenish the cancer cell reservoir. This grim scenario calls for novel strategies to target the anatomic homing of tumorigenic B cells. Here we have reported the targeting specificity and the therapeutic properties of an idiotype-specific peptide toward a murine B lymphoma engrafted in syngenic, immune competent mice. Our results show a highly-specific targeting of the selected A20 idiotype-specific peptide, which allowed the \textit{in vivo} detection of an early-stage of metastatic disease by using sensitive imaging techniques, such as fluorescence and MicroPET analysis (Fig. 3).

The \textit{in vitro} functional analysis of the Id-peptide used in this study underscores some relevant activities: a) the pA20-36 Id-peptide was specifically internalized by target cells with a kinetic shared with the surface A20-Ig upon BCR cross-linking, suggesting a mechanism of receptor-mediated endocytosis (Fig. S3); b) the pA20-36 Id-peptide triggered a BCR-mediated signalling that induced apoptosis of the target cells (Fig. 2). Accordingly, B cell apoptosis is observed in various types of both immature and mature neoplastic B cells following cross-linking of the sIg-BCR with surrogate antigens\(^{29}\). Moreover, effective immunotherapy of B-cell lymphoma based on passive transfer of Id-specific monoclonal antibodies correlated with anti-idiotype induced signal transduction in lymphoma cells\(^{30}\). The apoptotic pathway activated upon BCR-cross-linking has not been univocally clarified\(^{31}\); however, evidence for mitochondria as central executors of BCR-induced apoptosis, and caspases-2, -3, and -9 as effectors of the apoptotic program downstream of mitochondria was reported\(^{32-34}\). Our results indicate that the BCR oligomerization by multimeric presentation of Id-peptide, recruited the apoptosis-initiating protease caspase-9, which in turn activated the executioner proteases caspase-3 and -7, thus resulting in apoptotic cell death of the targeted tumor cells (Fig. 2D). Of interest, we also observed a significant apoptotic effect \textit{in vivo} in
the case of the monomeric Id-peptide (Fig. 4), suggesting that pA20-36 could acquire both monomeric and multimeric conformations. Although this possibility needs to be further investigated, previous studies have shown that receptor peptide ligands can mediate the dimerization of the cognate receptors; in support of this possibility, the crystal structure of a 20-residue cyclic mimetic peptide of erythropoietin (EPO) revealed a simple, compact homo-dimer of β-hairpin structure that induced cross-linking of the EPO receptor, resulting in signal transduction and cell proliferation\textsuperscript{35}.

Although cell apoptosis induced by chemotherapy or radiotherapy has been associated with a silent or tolerogenic immune response, recent studies have underscored the immunogenic properties of apoptotic cell death induced by chemotherapeutic compounds\textsuperscript{36-38}. In this study, we report that an A20 cell-specific Id-peptide induced a consistent anti-tumor immunity in immune competent mice harboring A20 B lymphoma cells. Indeed, we observed an increased number of armed CD8\textsuperscript{+} T cells that colonized the tumor masses in pA20-36-treated mice (Fig. 6D and 7A). Consistently, CD8\textsuperscript{+} T cells showed an A20 cell-specific proliferative and cytolytic activity (Fig. 7B and 7C). Consistently, the CD8\textsuperscript{+} T cell depletion in mice strongly counteracted the tumor inhibition by pA20-36, thus pointing to the relevant role for CD8\textsuperscript{+} T cells in the mechanism of tumoricidal activity mediated by pA20-36 \textit{in vivo}.

B-cell malignancies may present tumor antigen to the immune system by either a direct presentation\textsuperscript{39,40}, or by cross-presenting tumor antigens from apoptotic cells\textsuperscript{41}. We observed that \textit{in vivo} exposure of tumor cells to pA20-36 resulted in up-regulation of the co-stimulatory molecules CD80, CD86, and CD40 (Fig. 6B), which was restricted to the tumor microenvironment, since their expression on A20 cells upon \textit{in vitro} stimulation with pA20-36 was unaffected (Fig. S9). These observations underscore a role for neoplastic B-cells in presenting tumor antigens to T cells. Accordingly, a direct tumor-antigen presentation by A20 B-cell lymphomas resulting in a strong anti-tumor effect has been recently reported\textsuperscript{41}.
Tumor-specific CD8+ T cells from pCNT-treated mice lacked an effective cytolytic activity, indicating that the tumor microenvironment shifted toward a T-cell suppression. Such inactive CD8+ T cells have been isolated from tumors42,43, where local suppression of CD8+ T-cell activity was ascribed to the immunosuppressive cytokines TGF-β and IL-10 or Foxp3+ CD4+ regulatory T (Treg) cells44,45. Consistently, the A20 tumor model underscores some interesting properties: (i) tumor growth directly correlated with increased percentages of Treg cells infiltrating tumor, spleen, and tumor-draining lymph nodes46; (ii) Treg cells promoted an early immune escape mechanism in A20 tumor by suppressing the endogenous anti-tumor activity of both CD4+ and CD8+ T cells46. Although the mechanisms that mediate the reduced TGF-β, IL-10 and Treg upon Id-peptide treatment need further investigation, the immune analysis of A20 tumors indicates that the shift of the immune response in tumor microenvironment toward an immune activated status could account for the anti-tumor CD8+ T cell activity observed in pA20-36 treated mice. Further, our results highlight the property of fluorochrome- or [18F]-labelled Id-peptide to target in vivo both the tumor masses and micro-metastases, as assessed by histology and microPET analysis (Fig.3).

Of clinical interest, the selected idiotype peptide also exerted an effective killing activity in vivo. A major limitation of the Id-peptide-based approach is the requirement of a panning procedure for each individual patient. However, there is evidence that chronic antigenic stimulation may contribute to the neoplastic transformation and drives a selective process that shapes the antigenic repertoire of a subset of B-cell proliferation, including CLL47. In this setting, Id-peptides will provide a unique tool to define the antigenic specificities of the Igs isolated from tumor B-cells and may devise an effective peptide-based strategy.
ACKNOWLEDGEMENTS.

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AUTHORSHIP CONTRIBUTIONS

Contribution: C.P., designed and performed research, analyzed data, and wrote the paper; C.F. and E.I. performed research and assisted with data analysis; F.M.T., F.T and L.L. assisted with the FACS experiments; A.D.L., A.P., M.P., M.S. and E.V. assisted with RPLs construction and ELISA; M.R.P., M.L., S.G. performed microPET experiments; O.F. performed peptide synthesis; F.D.P. performed SPR experiments; N.C., C.A., A.G., G.P., A.B. assisted with the mouse experiments; M.G. performed MS analysis; I.Q. assisted with data analysis and reviewed the manuscript; G.S. conceived the study and wrote the paper.

DISCLOSURE OF CONFLICTS OF INTEREST

The authors declare no competing financial interests.
REFERENCES

### Table 1.

**Peptide ligands isolated from phage-display libraries.**

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<th>Phage clones</th>
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Peptide insert sequences of phage clones isolated from three RPLs by affinity purification with A20-Ig.

$^\text{A}$ELISA absorbance values of single phage clones were expressed as the difference between OD$_{405}$ nm and OD$_{620}$ nm.
Table 2.

In vivo toxicity of Id-peptides

<table>
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<th>IL1-β (pg/mL)</th>
<th>GSH (mM)</th>
<th>LDH (IU/L)</th>
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<td>Untreated</td>
<td>59±0.01</td>
<td>0.006±0.001</td>
<td>80.0±15.1</td>
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<tr>
<td>pCNT -treated</td>
<td>60±0.02</td>
<td>0.006±0.001</td>
<td>68.6±23.8</td>
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<tr>
<td>pA20-36 -treated</td>
<td>59±0.01</td>
<td>0.006±0.001</td>
<td>73.2±19.2</td>
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</table>

Three groups of tumor-free BALB/c mice (5 mice/group) were left untreated, or daily inoculated i.p. with either pA20-36-, or pCNT-peptide (20 mg kg⁻¹d⁻¹) for two weeks, after which blood samples were collected and analyzed for IL1-β, and GSH and LDH. Values represent the mean ± SEM of three different experiments.
FIGURE LEGENDS

Figure 1. *In vitro* specific targeting of A20-Ig peptide ligands.

(A) ELISA binding analysis of selected phages clones (left) or phage derived N-biotinylated synthetic peptides (middle) to purified A20-Ig. Peptide binding to polyclonal mouse immunoglobulins is also shown (right). Absorbance was calculated as the difference between OD_{405nm} and OD_{620nm}. Mean absorbance values ± SEM of four independent experiments are shown; RU, relative units. The wild type f88-4 phage, or a scrambled peptide (pCNT) was used as a control for the binding analysis of the selected phage clones or peptides, respectively. (B) Binding of FITC-conjugated peptides to A20 target cells by flow cytometry. Values are the mean fluorescent intensities (MFI) representative of two independent experiments (n=4). (C) Surface plasmon resonance analysis of the binding of the pA20-36 to A20-Ig. (D) Colocalization of FITC-conjugated pA20-36 peptide with the surface A20-Ig, as shown by confocal microscopy. Scale bar = 10 μm. In the diagram, the intensity profile for the pA20-36 and A20-Ig channel along a line scan through a representative cell is shown.

Figure 2. The pA20-36 peptide induces apoptosis of A20 cells in a caspase-dependent manner.

A20 cells (2 x 10^7/ml) were incubated with monomeric pA20-36 or pCNT peptide (20 μg/ml), or with the streptavidin-coated pA20-36 or pCNT peptide, and analyzed for viability, cell cycle profile and apoptosis. As positive control of apoptosis, cells were cultured in the presence of goat anti-mouse IgG F(ab)'2 (20 μg/ml). (A) Cell viability is strongly affected by streptavidin-coated pA20-36. Number of viable A20 cells was measured by trypan blue. Values are the mean ± SEM of three independent experiments; statistical analysis was performed by Student’s *t*-test. (B) The pA20-36 treatment increases sub-G1 (apoptotic) cell population. Histograms for cell cycle profiles of three independent experiments with similar results, as measured by PI staining and flow cytometry. (C)
The pA20-36 treatment induces apoptosis of A20 target cells. Dot plots for Annexin-V binding of three independent experiments with similar results. (D) The pA20-36 peptide induces activation of caspases. The activity of caspase-8, caspase-9 and effector caspase 3/7 was analyzed by luminometric assay of A20 cell extracts.

**Figure 3.** **pA20-36 specifically binds primary and metastatic A20 tumor cells.** (A) The pA20-36-FITC peptide specifically detects A20 tumor cells *ex vivo*. Cells (1x10⁶) derived from the site of s.c. injection (upper panels) or spleen (middle panels) from A20 B-lymphoma engrafted BALB/c mice at day 12 were incubated with FITC-conjugated pA20-36 or pCNT peptides (10 μg/ml), and PE-labeled anti-mouse B220 antibody, and analyzed by flow cytometry. Control splenocytes from tumor-free mice were also analyzed (lower panels). (B) The pA20-36-FITC peptide detects niches of metastatic A20 tumor cells in the spleen of A20 B-lymphoma engrafted BALB/c mice. Spleen from A20 B-lymphoma bearing mice were stained with pA20-36-FITC, anti-mouse B220 antibody, and the nuclear dye TOPO-3, and analyzed by confocal microscopy. (C) MicroPET images showing the specific retention of [¹⁸F]-pA20-36 in the A20 tumor. BALB/c mice bearing a palpable A20 tumor mass on the flank (5-6 mm in maximal diameter) were i.v. injected with 10 μg (8 MBq peptide) of [¹⁸F]-pA20-36 (left panels) or [¹⁸F]-pCNT (middle panels). Mice bearing a palpable 5T33MM tumor mass on the flank was used as control for the specific retention of pA20-36 (right panels). Images shown are coronal (upper panels) or axial (lower panels) sections of static scans of a single mouse for each group collected at 10, 30 and 60 minutes after peptide injection (see also Supplementary Videos online). Tumors are indicated by white arrows in all cases.

**Figure 4.** **pA20-36 inhibits tumor growth in vivo.** (A) Daily administration of pA20-36 results in inhibition of tumor growth in mice engrafted with A20 B-lymphoma. Tumor volumes were measured in 6-8 weeks old female BALB/c mice (n=5 per group) that had been s.c. injected with tumor cells (5x10⁶, left panel; 5x10⁵ middle panel), and treated with daily i.v. administration of
pA20-36, or scrambled pCNT (20 mg kg\(^{-1}\) d\(^{-1}\) in PBS), or left untreated, beginning the day after tumor cells injection. As control, tumor volumes were also measured in 6-8 weeks old C57BL/KaLwRij mice (n=5 per group) that have been s.c. injected with 5T33MM tumor cells (5x10\(^6\) right panel). One representative out of two independent experiments with similar results is shown. Values are the mean volumes ± SEM per group of animals (n=5/group). Statistical analysis was performed by two-way ANOVA. In case of large tumor cells load (5x10\(^6\)), \(P< 0.0001\) for pA20-36 group versus untreated; \(P< 0.0001\) for pA20-36 group versus pCNT group. In the case of small tumor cell load (5x10\(^5\)), \(P< 0.0001\) for pA20-36 group versus untreated; \(P< 0.0001\) for pA20-36 group versus pCNT group. (B) The pA20-36 treatment enhances survival of mice engrafted with A20 B-lymphoma. Kaplan-Meier survival curves of A20 B lymphoma-engrafted mice upon peptide treatments. BALB/c mice (n=5 per group) were s.c. inoculated with tumor cells and treated as described in A, and sacrificed when the tumor volume reached a size of 500 mm\(^3\), according to the Ethical guidelines. Statistical analysis was performed by Long-Rank Mantel-Cox test; statistically significant difference was observed between pA20-36-treated and pCNT-treated groups (\(P=0.0096\) for mice grafted with 5x10\(^5\) tumor cells, or \(P=0.0025\) for mice engrafted with 5 x 10\(^6\) tumor cells). One representative out of two independent experiments with similar results is shown. (C) Flow cytometry of apoptotic cells derived from pA20-treated and pCNT- treated mice. Apoptosis was evaluated at day 24 of peptide treatment by cell staining with Annexin V-FITC and PI. Apoptosis was measured as Annexin-V-positive/PI-negative cell population. Viable A20 cells from culture were used as control. A representative experiment out of four independent experiments is shown. Quantitative analysis of Annexin V-positive tumor cells from pA20-36- and pCNT- treated mice is also shown. Values are the mean ± SD (n= 4/group); statistical analysis was performed according to the Student’s \(t\)-test. (D) Cell apoptosis in tumor masses of A20 B lymphoma. Tissue sections of A20 tumor masses were labeled by TUNEL and anti-IgG to identify apoptotic DNA and B cells, respectively, and analyzed by confocal microscopy. Scale bar = 80 μm. (E) Cell cycle profiles of A20 and 5T33MM tumor cells after peptide challenge in vivo. To track tumor cell in vivo, A20 (left
pannels) or 5T33MM (right panels) cells were stained with CFSE, and i.p. injected in BALB/c mice. After three days, mice were i.p. injected with pCNT or pA20-36 peptide (100 μg/100 μl PBS). Six hours later mice were sacrificed and single cell suspensions were prepared from draining lymph nodes. Cell cycle profile was analyzed by flow cytometry on CFSE gated tumor population; data are the mean ± SD (n= 4/group); statistical analysis was performed according to the Student’s t-test.

Figure 5. Analysis of tumor microenvironment in A20 B lymphoma-engrafted mice. (A) Cytokine expression levels in tumor tissues from mice i.v. injected with pA20-36 or pCNT peptides (200 mg kg^{-1} d^{-1}) at day 24 post-treatment. Mean values ± SD of three independent experiments are shown. Statistical analysis was performed according to the Student’s t-test (n =4) tumors per group. (B) Inhibition of tumor angiogenesis by the pA20-36 peptide. Sections of fresh tumor tissues were incubated with antibody to CD31 to stain endothelial cells, together with TOPO-3 staining followed by confocal microscopy. Panels are representative of samples from four mice per group with similar staining profiles. (C) Quantitative analysis of vessel density. The mean number of vessels per field (56.25 μm²) of tumor sections described in B is shown; mean values ± SD (n=4/group); statistical analysis was performed according to the Student’s t-test.

Figure 6. Tumor infiltrating immune cells in pA20-36- or pCNT-treated A20 B lymphoma-engrafted mice. (A) The pA20-36 treatment affects the number of tumor-infiltrating CD4^+, CD8^+ T and Treg cells. Cell suspensions were prepared from tumor tissues at day 14 after s.c. tumor injection, and analyzed by flow cytometry upon staining with specific antibodies. Values are the mean ± SD (n= 4/group); statistical analysis was performed by Student’s t-test. (B) Tumor B220^+ cells show an increased expression of the activation markers upon pA20-36 treatment. Expression of activation markers in CD11c^+ (upper), CD11b^+ (middle) and B220^+ (lower) antigen presenting cells derived from tumor masses of pA20-36- or pCNT-treated A20 B lymphoma-engrafted mice as
assessed by flow cytometry. Histograms are representative of sample from five mice with similar staining profiles. (C) Tumor infiltrating CD8+ T cells from pA20-36-treated mice show an activated phenotype. Expression of activation markers in CD4+ (upper) and CD8+ T (lower) cells from tumor masses of pA20-36- or pCNT-treated A20 B lymphoma-engrafted mice as assessed by flow cytometry. Histograms are representative of sample from five mice with similar staining profiles. (D) Granzyme-positive CD8+ T cells infiltrating tumor masses of pA20-36- or pCNT-treated A20 B lymphoma-engrafted mice. Sections of fresh tumor tissues were incubated with antibody to CD8, anti-granzyme B, and with TOPO-3, followed by confocal microscopy. Scale bar = 14.2 μm.

**Figure 7. The CD8+ T activity significantly contributes to pA20-36 induced tumor growth inhibition.** (A) Intracellular cytokines expression of tumor infiltrating CD8+ T cells. Purified CD8+ T cells from pCNT- or pA20-36-treated tumor masses were incubated with A20 tumor cells for 3 h and then analyzed for intracellular expression of IL-2, IFN-γ and granzyme B by flow cytometry. A representative experiment out of two independent experiments with similar results is shown. (B) Antigen-specific proliferation of tumor infiltrating CD8+ T cells. Purified CD8+ T cells from tumor mass of pCNT- or pA20-36 treated mice were CFSE-stained, incubated with A20 or 5T33MM tumor cells (5x10^3), and one week later measured by flow cytometry. Percentage of proliferating CD8+ T cells was calculated from CFSE profiles using FlowJo software, as described in Materials and Methods. Values are the mean ± SD (n=3). A representative experiment out of two independent experiments with similar results is shown. (C) Cytotoxic activity of tumor infiltrating CD8+ T cells. Purified CD8+ T cells (effector cells, E) from pCNT- or pA20-36-treated tumor masses were incubated with BATD-labeled A20 or 5T33MM cells (5x10^3) (target cells, T), at ratio of 50:1 and 100:1. BATD-specific release was measured by time-resolved fluorometry. Maximal release of BATDA was measured by incubating target cells in lysis buffer containing 1% Triton X-100; spontaneous release was measured by incubating cells in medium alone. Cytolytic activity was calculated as: (experimental release - spontaneous release)/(Triton X-100 release x spontaneous...
release) x 100. Values are the mean ± SD (n=3); statistical analysis was performed by Student’s t-test. A representative experiment out of two independent experiments with similar results is shown.

(D) CD8+ T cells contributed to anti-tumor activity of pA20-36 peptide. BALB/c mice (n=6/group) were antibody-mediated depleted of CD8+ T cells, or left un-depleted, and s.c. injected with A20 tumor cells (5x10⁶); mice were then treated i.v. with pA20-36, or scrambled pCNT (200 mg kg⁻¹ d⁻¹), beginning the day after tumor injection. Tumor volumes were evaluated at the indicated time after tumor injection. A representative experiment out of two independent experiments is shown. Symbols represent the tumor volume of each individual mouse; horizontal lines indicate the mean. Statistical analysis was performed by two-way ANOVA.
Figure 3

A

Spleen from A20 lymphoma-bearing mice

B

Spleen from A20 lymphoma-bearing mice

C

s.c. A20 lymphoma + ^18^F-pA20-36

s.c. A20 lymphoma + ^18^F-CNT

s.c. 5T33MM + ^18^F-pA20-36

minutes from ^18^F-peptide injection
Figure 4

A. Tumor volume (cm³) over time for A20 and 5T33MM models with different treatments.

B. Mice survival (%) over time for A20 model with different treatments.

C. Flow cytometry analysis showing Annexin V-FITC positivity for A20 cell line and pCNT, pa20-36.

D. Immunofluorescence images showing TUNEL staining and Merge for A20 and 5T33MM treated with anti-IgG and pCNT or pa20-36.

E. Cell cycle analysis showing percentage of cells in different phases (G1, S, G2, >G1) for A20 and 5T33MM cells treated with pCNT or pa20-36.
Figure 5

A

Relative mRNA levels

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B

CD31

Nuclei

Merge

C

pCNT-treated mice vs. pA20-36-treated mice: microvessel density

P = 0.020
Figure 6

A

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B

CD11c+ cells

CD11b+ cells

B220+ cells

CD80

CD86

MHC II

PDL1

CD40

C

CD8+ T cells

CD4+ T cells

CTLA4

CD62L

PD1

CD28

D

CD8

Granzyme

Nuclei
In vivo targeting and growth inhibition of the A20 murine B-cell lymphoma by an idiotype-specific peptide binder

Camillo Palmieri, Cristina Falcone, Enrico Iaccino, Franca Maria Tuccillo, Marco Gaspari, Francesca Trimboli, Annamaria De Laurentiis, Laura Luberto, Marilena Pontoriero, Antonio Pisano, Eleonora Vecchio, Olga Fierro, Maria Rosaria Panico, Michele Larobina, Sara Gargiulo, Nicola Costa, Fabrizio Dal Piaz, Marco Schiavone, Claudio Arra, Aldo Giudice, Giuseppe Palma, Antonio Barbieri, Ileana Quinto and Giuseppe Scala