Exploiting a new strategy to induce immunogenic cell death to improve dendritic cell-based vaccines for lymphoma immunotherapy

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ABSTRACT

Although promising, the clinical benefit provided by dendritic cell (DC)-based vaccines is still limited and the choice of the optimal antigen formulation is still an unresolved issue. We have developed a new DC-based vaccination protocol for aggressive and/or refractory lymphomas which combines the unique features of interferon-conditioned DC (IFN-DC) with highly immunogenic tumor cell lysates (TCL) obtained from lymphoma cells undergoing immunogenic cell death. We show that treatment of mantle cell lymphoma (MCL) and diffuse large B-cell lymphoma (DLBCL) cell lines with 9-cis-retinoic acid and IFN\(_{\alpha}\) (RA/IFN\(_{\alpha}\)) induces early membrane exposure of Calreticulin, HSP70 and 90 together with CD47 down-regulation and enhanced HMGB1 secretion. Consistently, RA/IFN\(_{\alpha}\)-treated apoptotic cells and TCLs were more efficiently phagocytosed by DCs compared to controls. Notably, cytotoxic T cells (CTLs) generated with autologous DCs pulsed with RA/IFN\(_{\alpha}\)-TCLs more efficiently recognized and specifically lysed MCL or DLBCL cells or targets loaded with several HLA-A*0201 cyclin D1 or HLA-B*0801 survivin epitopes. These cultures also showed an expansion of Th1 and Th17 cells and an increased Th17/Treg ratio. Moreover, DCs loaded with RA/IFN\(_{\alpha}\)-TCLs showed enhanced functional maturation and activation. NOD/SCID mice reconstituted with human peripheral blood lymphocytes and vaccinated with autologous RA/IFN\(_{\alpha}\)-TCL loaded-IFN-DCs showed lymphoma-specific T-cell responses and a significant decrease in tumor growth with respect to mice treated with IFN-DC unpulsed or loaded with untreated TCLs. This study demonstrates the feasibility and efficacy of the use of RA/IFN\(_{\alpha}\) to generate a highly immunogenic TCL as a suitable tumor antigen formulation for the development of effective anticancer DC-based vaccines.

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ORIGINAL RESEARCH

Introduction

Non-Hodgkin lymphomas (NHL) include a heterogeneous group of lymphoproliferative disorders with widely varying biological, histological, and clinical features. Improvement in upfront immuno-chemotherapy and salvage therapy in recent years has resulted in prolonged disease control or cure in up to two thirds of patients.\(^1\) However, patients with relapsed or refractory disease have limited treatment modalities and new therapeutic strategies are urgently needed. Evidence accumulated so far indicates that the immune system is capable of immune surveillance and can be very effective against lymphomas. Moreover, results from early clinical trials, mainly including relapsed or refractory Diffuse Large B-cell Lymphomas (DLBCL), suggest that targeting the inhibitory immune checkpoint PD-1/PD-L1 axis may be a promising immunotherapeutic strategy for malignant lymphomas,\(^2\) although the response rates obtained so far are still unsatisfactory. Therefore, development of more effective immunotherapeutic strategies could improve the clinical benefit provided by current therapies for lymphoma patients. In the setting of relapsed or refractory NHL, immunotherapy could be particularly useful as a maintenance treatment aiming at boosting tumor-specific T-cell responses able to eradicate residual malignant cells thus preventing recurrence of the disease.

Among the high-grade NHLs, DLBCL and mantle cell lymphoma (MCL) represent appealing targets for immunotherapy because of their susceptibility to T-cell mediated immune effects, as demonstrated by long-term remissions after allogeneic stem cell transplantation.\(^4,5\) In particular, MCL cells overexpress cyclin D1, due to a t(11;14)(q13;q32) chromosomal translocation,\(^6\) and other tumor-associated antigens that may be successfully targeted by CD8\(^+\) and CD4\(^+\) T cells.\(^7,9\) In addition, in vivo studies demonstrated that adoptively transferred MCL-specific T cells were able to markedly inhibit tumor growth in mice with minimal residual MCL. Notably, when combined with CHOP chemotherapy, adoptive T-cell therapy was able to significantly extend...
the survival of lymphoma-bearing mice by further reducing the tumor burden. Moreover, a tumor cell-dendritic cell (DC) hybrid vaccine was shown to induce a complete clinical remission in a MCL patient. These findings are consistent with the possibility that cell-based immunotherapy may be effective in treating minimal residual disease in MCL and DLBCL, thus preventing lymphoma relapse.

A promising immunotherapeutic strategy relies on the use of cancer vaccines based on DCs, which are able to efficiently prime naïve T cells toward tumor antigens and elicit therapeutically relevant immune responses. As monotherapy, DC vaccination proved to be safe and rarely associated with immune-related adverse events, being thus an attractive therapeutic option for asymptomatic patients. Nevertheless, the choice of the optimal antigen formulation to be used for DC loading is still an open issue and constitutes one of the main factors limiting the potency of DC-based vaccines. Strategies aiming at enhancing the immunogenicity of dying or dead cancer cells used as a source of antigens may improve the therapeutic potential of DC-based cancer vaccines. Indeed, the in vivo efficacy of DC vaccines can be significantly influenced by the methodology used to prepare the tumor cell cargo, as shown in multiple preclinical cancer models. In particular, recent evidence highlighted the therapeutic relevance to exploit the concept of immunogenic cell death (ICD) to obtain highly immunogenic antigen sources for the development of “next-generation” DC-based immunotherapy. In fact, necro-inflammatory cells undergoing ICD showed superior immunogenicity being able to promote strong antitumor responses largely biased toward Th1 immunity. On these grounds, we have developed a new DC-based vaccination protocol for aggressive and/or refractory lymphomas which combines the unique features of interferon-conditioned DC (IFN-DC), with highly immunogenic tumor cell lysates (TCL) obtained from lymphoma cells undergoing ICD induced by 9-cis-retinoic acid and IFNα (RA/IFNα), a drug combination that we herein show to induce a strongly immunogenic apoptosis. Different options of defined cell culture conditions are available for generating relatively large numbers of DCs from cell precursors. We chose a particularly effective modality of ex vivo differentiation of DCs from human monocytes consisting in a single step 3-day culture in the presence of GM-CSF and IFNα. The DCs generated by this method, designated IFN-DC, exhibit a phenotype of highly active, partially mature DCs, endowed with a high migratory behavior and immuno-stimulatory ability. Several in vitro and in vivo studies conducted in immunodeficient SCID mice reconstituted with human peripheral blood lymphocytes have shown that antigen-pulsed IFN-DC can improve human immune responses toward both viral and tumor antigens. The results presented herein demonstrate that exploitation of a novel modality to induce ICD allows the generation of highly immunogenic tumor cell lysates which may improve the therapeutic potential of DC-based vaccines for refractory or relapsed NHLs.

Results

RA/IFNα combination induces ICD in MCL and DLBCL cell lines

We have previously shown that RA/IFNα treatment induces marked apoptotic responses in MCL cells by up-regulating the pro-apoptotic protein Noxa. Similar findings were also observed in the DOHH2 DLBCL cell line (Figure 1A, B) indicating that the pro-apoptotic effects of RA/IFNα also extend to other aggressive B-cell NHLs. The observation that the ICD induced by chemotherapeutic agents, such as doxorubicin, is strictly dependent on the activation of type-I IFN pathway prompted us to assess the immunogenicity of RA/IFNα-induced MCL cell apoptosis. To this end, we investigated the effect of this treatment on established ICD-associated markers. As shown in Figure 1C, multispectral imaging flow cytometry gating on viable cells showed at single cell level that RA/IFNα treatment significantly enhanced calreticulin (ecto-CRT) exposure and down-regulated the CD47 phagocytosis inhibitor in all lymphoma cell lines investigated (Figure 1C). These findings are in line with the existence of an inverse correlation between CD47 and ecto-CRT expression, which contributes to generate favorable conditions for the uptake of apoptotic tumor cells by DCs. The statistically significant increase in ecto-CRT was confirmed by classical flow cytometry in at least three independent experiments (Figure 1D-E). Notably, spot count assessed by the IDEAS software indicated that the extent of ecto-CRT expression (number of spots) significantly increased dependently on treatment (Supplementary Figure S1A). Translocation of CRT is the consequence of the induction of an ER stress response characterized by the phosphorylation of the translation initiation factor eIF2-α (p-eIF2-α). Consistently, RA/IFNα treatment significantly increased p-eIF2-α levels in Mino and SP53 cells (Supplementary Figure S1B). RA/IFNα treatment was also shown to enhance cell surface expression of heat shock proteins (HSPs) 70 and 90 in Mino, SP53, and DOHH2 cells (Figure 2A). Analysis of the release of high-mobility group box 1 (HMGB1) as a late event in the ICD process showed that only after 72 h of treatment a significant increase of HMGB1 levels was observed in the culture supernatants of all three RA/IFNα-treated lymphoma cell lines (Figure 2B). Consistently, these results indicated that RA/IFNα treatment of MCL and DLBCL cells in vitro induces the main phenotypic and immunologic markers currently used to define the occurrence of ICD.

To confirm the immunogenicity of RA/IFNα-induced apoptosis in an in vivo model of lymphoma, we used the A20 mouse lymphoma cells. In keeping with what observed in human lymphomas, treatment of A20 cells with RA and mouse IFNα induced a significant CRT exposure coupled with CD47 downregulation (Supplementary Figure S2A). Notably, Balb/c mice inoculated s.c. with RA/IFNα-treated A20 cells as a tumor vaccine were protected against a subsequent challenge with live A20 cells injected in the opposite flank (Supplementary Figure S2B). Similar findings were observed in mice vaccinated with A20 cells treated with the ICD inducer doxorubicin (Supplementary Figure S2B).

Tumor cell lysates derived from RA/IFNα-treated lymphoma cells as suitable tumor antigen source for DC-based vaccines

Given the ability of RA/IFNα to induce immunogenic apoptosis and the promising results deriving from the application of ICD inducers to DC vaccines in different preclinical and clinical studies, we exploited RA/IFNα-induced ICD to
obtain an optimized source of antigens for improved DC-based vaccine. To boost a rapid GMP upgrading, we preferred the use of whole tumor cell lysates (TCLs) obtained from lymphoma cells undergoing ICD instead of using apoptotic cells or bodies. Indeed, TCLs are more accurately quantifiable, easier to handle and better preservable in frozen state than the starting tumor cells. Moreover, taking into account that a complete devitalization of tumor material is required before DC pulsing, repeated freeze/thawing cycles were used to achieve the absolute absence of living cells in the TCL and a complete lysis at the same time. The presence of ICD-related immunogenic markers was verified in TCLs from RA-IFNα-treated cells and compared to TCLs of γ-irradiated or untreated cells. As shown in figure 3A, RA-IFNα-TCLs, obtained after 5 days of treatment and following three freeze/thawing cycles from both MCL and DLBCL cell lines displayed higher expression of CRT and HSP70 with respect to untreated- and γ-irradiated-TCLs. Moreover, only in RA-IFNα-TCLs an increase of the cleaved form of caspase 3 was detected, in keeping with the pro-apoptotic effects of the treatment. In contrast, HMGB1 protein expression levels decreased in RA/IFNα-TCLs consistent with a higher release of this alarmin in the cell supernatants of treated cells (Figure 3A). Conversely, the amount of ATP was increased in treated TCLs, albeit the difference was statistically significant only in one (Mino) out of the two cell lines investigated (Figure 3B). Taking advantage from multispectral imaging flow cytometry, we assessed the extent of RA/IFNα-treated and untreated cell or TCL uptake by DCs. To this end, Mino and DOHH2 cell lines were labeled with the tracer Fast DiA and then treated or not for 5 days. The last day, tumor cells or TCLs were used to pulse CD11c-labeled differentiated DCs. Notably RA/IFNα-treated apoptotic cells and RA/IFNα-TCLs were more efficiently recognized and phagocytosed by DCs compared to untreated ones (Figure 3C-D). These findings supported the rationale for the use of RA/IFNα-TCLs as a suitable antigen formulation for DC-based vaccine development.

Figure 1. RA/IFNα combination induces ICD. A-B) Mino, SP53 and DOHH2 cells were cultured in the absence or presence of RA/IFNα for five (A) or three (B) days. (A) The percentage of early (grey, annexinV-positive and Propidium iodide-negative (AnnexV−)) and late (white, both annexin-V- and propidium iodide-positive (AnnexV−PI−)) apoptotic cells is shown. Bars, mean of at least three independent experiments; error bars, SD (‘p < 0.05 Student’s t-test). (B) Immunoblotting analysis for the indicated proteins. A representative experiment was reported. (C) Mino, SP53 and DOHH2 cells were cultured in the absence or presence of RA/IFNα for 48 hours. 10⁶ cells/sample were labeled with primary antibodies against CRT and CD47 and acquired with ImageStreamX. The SYTOX® AADVanced™ Dead Cell Stain (7-AAD) was used to exclude dead cells from the analyses. Fluorescence histograms (top) of a representative experiment is shown. Filled curves refer to untreated samples, empty curves to RA/IFNα-treated ones. At the bottom, exemplary images of CRT/CD47 staining showing an inverse correlation between CRT and CD47 expression induced by RA/IFNα treatment. (D-E) ecto-CRT exposure analyzed by classical flow cytometry. Bars, mean of three independent experiments; error bars, SD (‘p < 0.05 **p < 0.01 Student’s t-test).
Figure 2. RA/IFNα combination promotes HSPs exposure and HMGB1 release. (A) Flow cytometry evaluation of HSP70 and HSP90. Gating strategy on 7-AAD negative cells allowed to exclude dead cells. Exemplary plots were shown on the left and histogram graphs of three independent experiments were shown on the right: bars represent mean and error bars SD. (*p < 0.05; **p < 0.01 Student’s t-test) (B) Early (24 h) and late (72 h) release of HMGB1 assessed by ELISA assay in Mino, SP53 and DOHH2 supernatants. Bars, mean of three independent experiments; error bars, SD. (*p < 0.05 Student’s t-test).

Figure 3. Tumor cell lysates as antigen source for development of DC-based vaccine. (A). Mino, SP53 and DOHH2 cells were cultured in the absence or presence of RA/IFNα for 5 days or exposed to γ-radiations. Then whole-cell lysates were obtained through three freeze/thaw cycles and separated by SDS-PAGE. Immunoblotting analysis of ICD markers was performed and GAPDH was used as loading control. Data are representative of 1 of 2 independent experiments (B) ATP levels were evaluated in Mino and DOHH2 lysates. Bars, mean of 3 independent experiments; error bars, SD. (*p < 0.05 Student’s t-test). (C-D) IFN-DCs were obtained from healthy donor-derived monocytes as explained in Methods. Tumor cell lines were labeled with the tracer Fast DiA and treated for 5 days with RA/IFNα. Treated or untreated tumor cells (top), and the respective TCLs (bottom), were co-culture with IFN-DCs for 4 hours, then cells were harvested and labeled with anti-CD11c. The percentage of phagocytic DCs (CD11c+/Fast DiA−) was evaluated by multispectral imaging flow cytometry. (C) A representative experiment was reported. (D) Phagocytosis assay was performed in duplicate from three different healthy donors. Bars, mean of three unrelated healthy donors; error bars, SD. (*p < 0.05).
**DCs loaded with RA/IFNα-treated TCLs elicit enhanced tumor-specific T-cell responses**

Immunogenicity of DCs loaded with RA/IFNα-TCLs derived from different lymphoma cell lines was investigated by analyzing the extent and specificity of the lytic activity of T-cell cultures generated from HLA-matched donors after co-culture with autologous DCs pulsed with MCL-derived TCLs. For these experiments, we exploited the enhanced immunogenic properties of IFN-DCs, which were pulsed with TCLs from live (untreated/CNTR), apoptotic (RA/IFNα-treated) and necrotic (γ-irradiated) MCL and DLBCL cells and then used to generate autologous lymphoma-specific T lymphocytes. Notably, IFN-DCs loaded with RA/IFNα-TCLs proved to be more immunogenic than IFN-DCs pulsed with CNTR- and γ-irradiated-TCLs. As shown in Figure 4A-B, CTL cultures generated with autologous RA/IFNα-TCL loaded-

![Figure 4](image-url)

**Figure 4.** RA/IFNα-TCL pulsed-IFN-DCs induce tumor-specific CTLs with enhanced killing efficiency. (A) HLA-A*0201-restricted healthy donor-derived lymphoma-specific CTL cultures were able to efficiently lyse MCL cell lines. The incubation of target cells with the anti-HLA A*0201 cr11.351 mAb prevented almost completely the lysis mediated by CTLs primed with RA/IFNα-TCL pulsed-DCs. Only background lysis was observed for CTLs cultured with unpulsed-DCs, CNTR-TCL or γ-radiated-TCL pulsed-DCs in all performed tests. (p < 0.05, Student’s t-test). (B) HLA-B*0801-restricted tumor-specific CTLs from different healthy donors were generated with IFN-DCs loaded either with CNTR-TCLs, RA/IFNα-TCLs and γ-rad-TCLs obtained from DOHH2 DLBCL cell line. (p < 0.02, Student’s t-test). (C) HLA-A*0201 healthy donor-derived tumor specific CTLs were able to specifically recognize and lyse autologous cells presenting 5 HLA-A*0201-restricted cyclin D1-derived peptides. Peptide-loaded T2-A2 cells were used as target cells and CTL cultures were obtained co-culturing (left) Mino cell line lysate-loaded and (right) SP53 cell line lysate-loaded IFN-DCs with autologous T lymphocytes. (p < 0.05, Student’s T-test). (D) HLA-B*0801 healthy donor-derived tumor-specific CTLs were able to specifically recognize and kill autologous EBV-LCLs presenting several HLA-B*0801-restricted Survivin-derived peptides. Peptide-loaded autologous EBV-LCLs were used as target cells and CTLs were generated by co-culturing DOHH2 TCL-loaded IFN-DCs with donor’s T lymphocytes. All cytotoxicity assays, evaluated by standard calcein-AM release assay, were performed after 4 re-stimulations by using as target cells either TCL-corresponding tumor cell lines and peptide-loaded T2-A2 cells/autologous LCLs. The graphs represent a mean of 3 independent experiments from different healthy donors. All tests were performed in triplicate at E:T ratio of 10:1, when tumor cell lines were used as targets, and at E:T of 20:1 in peptide-specific cytotoxicity assays. ( * vs unpulsed-DCs; ** vs CNTR-TCL pulsed-DCs).
IFN-DCs exerted a significantly higher and more efficient cytotoxic activity against the cell lines used for CTL priming, in a HLA-restricted fashion, as compared to unpulsed-DCs or DCs loaded with control TCLs (Figure 4A-B). Only basal levels of specific cytotoxicity responses were observed against K562 cell line, thus excluding non-specific NK-like cytotoxicity for all examined CTL cultures (Supplementary Figure S3A). Notably, blocking CR1 with a specific antibody during DC pulsing with RA/IFNα-TCLs inhibited the enhanced efficiency of ICD-TCL pulsed-DCs to priming tumor specific CTLs, demonstrating the contributory role of this DAMP in our system (Supplementary Figure S3B).

Moreover, CTLs derived from RA/IFNα-TCL pulsed-IFN-DCs induced significantly higher cytotoxic responses against several HLA-A*0201-restricted cyclin D1-derived epitopes, as compared to control T-cell cultures, suggesting an enhanced processing and/or presentation of tumor-associated antigens/epitopes (Figure 4C). Intriguingly, similar results were obtained by the analysis of peptide-specific CTL responses against HLA-B*0801-restricted epitopes derived from the universal tumor-associated antigen Survivin when RA/IFNα-TCLs from DOHH2 cell line were used to load IFN-DCs (Figure 4D).

**ICD-TCL pulsed-DCs increased Th1 and Th17 cells**

An ideal cancer vaccine should be able to trigger potent CTL and Th1-driven antitumor responses and, at the same time, to inhibit immunosuppressive cells, such as regulatory T cells (Tregs). Therefore, to gain insights on the mechanisms underlying the enhanced immunogenicity of RA/IFNα-TCL pulsed DCs, we characterized the T-cell subpopulations obtained during ex vivo CTL generation. T-cell cultures were obtained from three healthy donors and different markers and intracellular cytokines were investigated after the first and the fourth/final cycle of stimulation with TCL-loaded DCs. At the end of the stimulation protocol, RA/IFNα-TCL pulsed DCs enhanced the fraction of CD4+IFNγ+/TNFα- cells in all four cultures investigated (Figure 5A). Consistently, in the same cultures, RA/IFNα-TCL pulsed DCs substantially increased the number of Th17 cells (Figure 5B), although no statistical significance was highlighted as a likely consequence of the high inter-donor variability. Interestingly, in all conditions, a significant decrease of Treg fraction was promoted during CTL generation, as shown in figure 5C, where the number of Tregs after both first and fourth stimulation was reported. Moreover, unlike what was observed in control cultures, RA/IFNα-TCL pulsed-DCs increased the Th17/Treg ratio (Figure 5D) tilting the balance between immune effectors and immunosuppressive cells toward the first, consistent with the possible generation of efficient T-cell mediated antitumor responses.

**ICD-TCLs enhance DC maturation and activation**

The higher efficiency of DCs loaded with RA/IFNα-TCLs in the induction of tumor- and antigen-specific CTLs prompted us to investigate in more detail the effects of these TCLs on DC maturation and activation. RNA-Seq analysis allowed the identification of 1,711 mRNAs differentially expressed in RA/IFNα-TCL and CNTR-TCL pulsed-DCs compared to unpulsed-DCs (FDR ≤ 0.05 and FC greater than 1.5-fold in at least 1 comparison). In particular, RA/IFNα-TCLs obtained from Mino and DOHH2 shared 407 mRNAs (Figure 6A). Functional annotation analysis of the differentially expressed transcripts indicated an enrichment of several canonical pathways most significantly affected by RA/IFNα-TCL pulsing (Figure 6A). In particular, the more strongly activated pathways in RA/IFNα-TCL pulsed-DCs included Dendritic Cell Maturation, IL-6 Signaling and Toll-Like Receptor Signaling directly associated with the maturation/activation status of DCs, and the HMGB1 Signaling probably related to ICD. Immunophenotypic analyses by flow cytometry was then performed to assess the possible contribution of RA/IFNα-TCL pulsing to IFN-DC maturation. The analysis showed that RA/IFNα-TCLs did not affect the expression levels of classical maturation markers, such as CD80/CD86, CD83 and MHC class II (HLA-DR), likely because the use of GM-CSF and IFN-α in the DC differentiation protocol already provides an adequate stimulus for the expression of these molecules (Supplementary Figure S4). Therefore, we investigated other features associated with DC maturation, such as the decrease of antigen-capture activity and the ability to secrete different cytokines. As depicted in Figure 6B, RA/IFNα-TCL pulsed-DCs showed a reduced receptor-mediated endocytosis in the presence of the fluorescent reporter DQ-OVA, consistent with an enhanced functional maturation. With regard to cytokine production, transcriptome analysis showed in particular the up-regulated activation of IL-6 signaling. Assessment of the secretion of pro-inflammatory cytokines by DCs after overnight pulsing with different TCLs disclosed a stronger production of IL-6 and TNFα, and slightly higher secretion of IL-1β and IL-10 by DCs loaded with RA/IFNα-TCLs compared with unpulsed- or CNTR-TCL pulsed-DCs (Figure 6C). Undetectable levels of IL-10 (data not shown) were observed in all experimental conditions consistently with the lack of Treg expansion (Figure 5C).

Next, given that the activation of Toll-Like Receptor pathway culminates in NF-κB signaling stimulation and pro-inflammatory cytokine production, we explored the ability of RA/IFN-α-TCLs to induce the activation of NF-κB in DCs. Analysis of the nuclear translocation of the NF-κB subunit p65 by multispectral imaging flow cytometry demonstrated that RA/IFN-α-TCLs derived from both Mino and DOHH2 cell lines promoted a significant shift of the p65 protein from cytoplasm into the nucleus (Figure 6D-E) in a higher proportion of cells. These data indicated that the loading with RA/IFNα-TCLs improves maturation and activation of IFN-DCs and enhances their efficacy to elicit tumor- and antigen-specific immunity.

**RA/IFNα-TCL pulsed-DC vaccine inhibits lymphoma growth in vivo**

The in vivo therapeutic potential of our DC vaccine was assessed in immunodeficient NOD/SCID mice reconstituted with human peripheral blood lymphocytes (hu-PBL-NOD-scid mice) and vaccinated with human autologous TCL loaded-IFN-DCs. We preliminarily assessed the ability of RA/IFNα-TCL pulsed-IFN-DCs to stimulate tumor specific T-cell responses. CD3+ T cells were recovered from spleen following three cycles of vaccination (Figure 7A) with Mino RA/IFNα-TCL or CNTR-TCL-pulsed- or unpulsed-IFN-DCs. As shown in
Figure 7A, mice immunized with IFN-DC pulsed with Mino RA/IFNα-TCLs showed higher numbers of T cells secreting IFNγ and granzyme-B when re-stimulated with Mino cells and analyzed by ELISPOT assay (Figure 7A).

Thus, to support the clinical feasibility of the developed vaccine, we tested the efficacy of RA/IFNα-TCL pulsed-DC vaccination in a therapeutic setup using a hu-PBL-NOD-scid mouse model. Preliminary experiments demonstrated that subcutaneous inoculation of 5 × 10⁶ Mino cells originated a tumor mass that began to grow after fourteen days since implantation. During that period, mice were reconstituted with human PBLs and subsequently exposed to three cycles of vaccination (Figure 7B Therapeutic vaccination schedule). We compared three groups of mice: the control group with unpulsed IFN-DC, a group with IFN-DC pulsed with CNTR-TCLs and the third group composed of mice vaccinated with RA/IFNα-TCL pulsed-DCs. Mice vaccinated with RA/IFNα-TCL pulsed-DCs experienced a significant (p < 0.05) decrease in tumor growth with respect to the other two groups (Figure 7B). All mice survived until sacrifice with no changes in any vital organs among the different treatment groups (data not shown). Notably, we observed a slight reduction (Figure 7C) (not statistically significant) in weight in mice vaccinated with RA/IFNα-TCL pulsed-DCs. Of interest, high levels of IFNγ were measured in the serum of mice vaccinated with RA/IFNα-TCL pulsed-DCs consistent with the activation of a Th1-driven response (Figure 7E). The production of other pro-inflammatory cytokines, such as IL-6 and TNFα, was not detected in all three groups of vaccinated mice, whereas the levels of IL-1β and PGE2 were not significantly different in RA/IFNα-TCL pulsed-DC vaccinated mice with respect to the other groups (Supplementary Figure S5).

Overall, the in vivo results supported the clinical benefit and therapeutic applicability of RA/IFNα-TCL-DC therapeutic vaccine as a new immunotherapeutic approach in aggressive lymphoma management.

Discussion

In the last years, the concept of ICD has overturned the dogma describing apoptosis as an immune-silent type of cell death. Moreover, the idea to combine ICD with tumor vaccines recently brought encouraging results for the treatment of both hematologic and solid tumors. Indeed, several studies have demonstrated that the use of ICD-experiencing cancer cells as source of tumor antigens may allow the development of highly efficient DC-based vaccines in different tumor models. This strategy exploits the advantages of treating tumor cells with ICD-inducers ex vivo, including drugs not currently used in cancer management or the use of...
Figure 6. RA/IFN-α-TCLs improve IFN-DC maturation and activation. (A) Venn showing 1,711 mRNAs, among which 407 are in common between Mino- and DOHH2-derived TCLs, differentially expressed in RA/IFN-α-TCL pulsed-IFN-DCs compared to INF-DCs loaded with CNTR-TCLs (FDR < 0.05 and FC greater than 1.5-fold in at least 1 comparison) by RNA-Seq analysis. Functional annotation analysis (on the left) of the differentially expressed transcripts indicates an enrichment (activation z-score > 2) of several canonical pathways most significantly affected in RA/IFN-α-treated TCL-loaded IFN-DCs such as Dendritic Cell Maturation, Toll-like Receptor Signaling and IL-6 Signaling. (B) IFN-DCs from 2 different healthy donors were loaded or not with different TCLs, derived from both Mino and DOHH2 cells. Successively were exposed to DQ-OVA and antigen uptake was evaluated at the indicated time-points by ImageStreamX technology. Minimal non-specific binding of DQ-OVA to the cell surface was determined by incubating samples at 4°C. Bars, mean; error bars, SD (Student’s t-test). (C) The effects of different TCLs used for IFN-DC pulsing on cytokine secretion were analyzed by ELISA assay. TNF-α and IL-6 were evaluated about 16 hours after the addition of TCLs, IL-1α and IL-1β after 30 hours. Graphs represent the mean of at least 3 independent experiments from different healthy donors. (p < 0.05, Student’s t-test). (D-E) NF-κB nuclear translocation was evaluated by multispectral imaging flow cytometry through the Similarity Score (SS) algorithm. Each plot represents NF-κB/DRAQ5 SS and the reported value indicates the percentage of DCs with NF-κB translocated into the nucleus (D) ML-1 cell line untreated or treated with TNF-α was used as negative and positive control, respectively, for defining the threshold of SS to consider translocated NF-κB. (E) Plots of an exemplary experiment were shown. Graph at the bottom represents the mean of 3 independent experiments. (p < 0.05, Student’s t-test).
conventional chemotherapeutics at higher concentrations than those tolerated in vivo. More important, the direct application of ICD features in developing a DC-based vaccine offers the benefit to markedly boost host immune system through an active tumor-specific immunotherapeutic approach. In line with these considerations, herein we propose the use of RA/IFNα combination as a novel and highly effective modality to induce ICD ex vivo in MCL and DLBCL cells and generate a highly immunogenic TCL as improved antigen source for DC loading. For the in vivo assessment of the therapeutic efficacy of our vaccine, we chose IFN-conditioned DCs on the basis of their capacity to release a unique array of cytokines and chemokines, known to efficiently favor Th1-type responses, and their enhanced capacity to stimulate CD8+ T-cell immunity. Moreover, IFN-DCs are competent in preserving internalized proteins from early degradation and in routing antigens toward the MHC-I processing pathway, allowing long-lasting cross-priming capacity.

In the present study, we identified RA/IFNα combination as a novel treatment capable to induce ICD in MCL and DLBCL cells and generate a highly immunogenic TCL as improved antigen source for DC loading. For the in vivo assessment of the therapeutic efficacy of our vaccine, we chose IFN-conditioned DCs on the basis of their capacity to release a unique array of cytokines and chemokines, known to efficiently favor Th1-type responses, and their enhanced capacity to stimulate CD8+ T-cell immunity. Moreover, IFN-DCs are competent in preserving internalized proteins from early degradation and in routing antigens toward the MHC-I processing pathway, allowing long-lasting cross-priming capacity. The feasibility and safety of IFN-DC-based vaccines have been evaluated in animal models and in a phase I clinical trial in advanced melanoma. Notably, Lapenta C. et al. have recently demonstrated the practicability of the approach based on IFN-DC loaded with autologous apoptotic follicular lymphoma cells. Our findings demonstrated the ability of the highly immunogenic RA/IFNα-TCLs to activate IFN-DCs and make them more efficient in eliciting tumor and antigen-specific CTLs in vitro and in mediating tumor growth inhibition in vivo, compared with IFN-DCs loaded with lysates from untreated or γ-irradiated/necrotic cells. In particular, the analysis of T-cell

**Figure 7.** Generation of specific human CD3+ T cells in hu-PBL-NOD-scid mice immunized with TCL pulsed-DCs and evaluation of tumor-growth inhibition. (A) Immunization schedule for hu-PBL-NOD-scid mice vaccination. Mice were injected i.p. with 40 × 10^6 PBL from HLA-A2 healthy blood donors. Four days after reconstitution, hu-PBL-SCID mice were injected i.p. with autologous IFN-DC loaded with TCL (200 μg). Vaccinated mice received boost immunizations after 7 and 14 days and were sacrificed 7 days later. Evaluation of CD3+ T cell response by ELISPOT assay for IFN-gamma and Granzyme B. Human cells recovered from four spleens of hu-PBL-SCID mice from each group were pooled. The assay was performed using as stimulators MCL cells Mino. Bars represent the CD3+ T cell response from hu-PBL-NOD-scid mice immunized with either RA/IFNα-TCL pulsed-DCs or CNTR-TCL pulsed-DCs as compared to the basal CD3+ T cell response from hu-PBL-NOD/SCID mice injected with unloaded IFN-DCs. Bars represent the mean ± SD of 3 independent experiments. (B) Evaluation of tumor-growth inhibition after therapeutic vaccination schedule. Graphs represent Mino cell growth rate in hu-PBL-NOD-scid mice immunized as described in materials and methods (p < 0.05 Student’s t-test). (C) Evaluation of therapeutic vaccination effect on body weight in tumor-bearing hu-PBL-NOD-scid mice. Data represent the mean body weight in the different treatment group. One representative experiment is shown. (D) Assessment of IFNγ levels in serum of mice after therapeutic vaccination schedule (p < 0.05 Student’s t-test).
subpopulations stimulated ex vivo by priming with RA/IFNα-TCLs pulsed-DCs suggested a likely contribution of Th1 and Th17 cells in mediating anti-tumor immune responses. Moreover, the high levels of IFNγ detected in sera of treated mice is consistent with the ability of RA/IFNα-TCLs pulsed-DCs to induce a systemic Th1-skewed immune response. Our results are in agreement with the recently reported observation that shikonin treated-TCLs enhanced DC maturation and differentiation of Th1 and Th17.32 Likewise, Garg AD et al. recently described an efficient Th1 immunity-biased DC immunotherapeutic approach in high-grade glioma exploiting the hypericin-based photodynamic therapy as ICD inducer.20

With regard to the mechanisms underlying the immunologic effects exerted by RA/IFNα TCLs on DCs, we have demonstrated that DCs loaded with these lysates show early enhanced activation of the NF-kB pathway and consequent secretion of pro-inflammatory cytokines. Interestingly, functional annotation analysis of the differentially expressed DC transcriptome indicated a deactivation of the NF-kB pathway driven by the up-regulation of different inhibitory molecules. These results may support the triggering of an inflammatory response by RA/IFNα TCL-DCs for a limited period of time, fundamental feature in order to avoid that the uncontrolled inflammation could become pro-tumorigenic and able to mediate local and systemic toxicity.

Our findings further support the relevance of type I IFN-mediated immunologic effects on tumor cells and the surrounding microenvironment in eliciting therapeutically effective antitumor immune responses. Recent evidence in fact indicates that the therapeutic effects mediated by anthracyclines, known ICD inducers, are mainly mediated by autocrine and paracrine circuitries triggered by IFN-α. Moreover, a type I IFN-related signature was shown to predict clinical responses to anthracycline-based chemotherapy in several independent cohorts of patients with breast carcinoma.46 Consistently, our results indicate that ex vivo treatment with RA/IFNα markedly enhances the immunogenicity of apoptotic lymphoma cells, which are also more easily taken up by DCs. This strategy appears particularly relevant to reprogram the immunogenic features of tumor cells ex vivo in order to obtain more potent TCLs for improved DC-based vaccination. Retinoids are known to enhance the pro-apoptotic effects of type I IFN and modulate several immune functions.47 Further studies are however required to better understand the cellular basis underlying the enhanced immunogenicity of RA/IFNα-treated lymphoma cells.

It is worth noting that the efficacy of RA/IFNα TCL-DCs was demonstrated in both DLBCL and MCL cell lines, indicating that this vaccination strategy may have a broad applicability as a consolidation therapy for different types of aggressive lymphomas. Combination with other immunotherapeutic approaches may also represent an additional setting in which our DC-based vaccination could be investigated. In fact, available data indicate that combinations of cancer vaccines with checkpoint inhibitors may lead to synergistic effects and higher response rates than monotherapy.48

In conclusion, the use of ICD inducers ex vivo, and particularly our RA/IFNα combination, allows to overcome the limitation of the toxicity related to the high doses administered in vivo to obtain the desired therapeutic effects. This strategy allows a full exploitation of the immunotherapeutic properties of ICD, thus maximizing its potential for clinical application. The present study demonstrates the feasibility and efficacy of the use of RA/IFNα to generate a highly immunogenic TCL as a suitable tumor antigen formulation for the development of effective anticancer DC-based vaccines. Our results provide the rationale for the design of a phase I/II clinical trial aimed at assessing the safety, immunogenicity and efficacy of our RA/IFNα TCL-DCs in patients with relapsed or refractory DLBCL or MCL.

Materials and methods

Cell lines

Mino, SP53 and Granta 519 MCL cell lines, DOHH2 DLBCL cell line, T2-A2, transporter-associated with antigen-processing-deficient T2 cells stably transfected with the HLA-A*0201 class-I molecule, EBV-transformed lymphoblastoid B-cell lines (EBV-LCLs), generated after the immortalization of healthy donor’s B-lymphocytes with the marmoset-derived B95.8 EBV strain and the mouse A20 lymphoma cell line were used in this study.

Cells were cultured in RPMI 1640 or DMEM (Granta 519) supplemented with 10% FCS (Fetal Calf Serum) and 100 μg/mL streptomycin, and 100 IU/mL penicillin (Sigma-Aldrich) and maintained at 37°C in a humidified 5% CO2 atmosphere.

Antibodies and reagents

HSP70 (#4872), HSP90 (#4874), p-eIF2-α (#9721), eIF2-α (#9722), cleaved caspase-3 (#9661) antibodies were from Cell Signaling Technology, PARP-1 (SC8007) and tubulin (SC9104) antibodies were from Santa Cruz Biotechnology; human CRT strain and the mouse A20 lymphoma cell line were used in this study.

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and HSP90 (ADI-SPA-830-488) were from Enzo Life Science, anti-rabbit-PE (111-116-144) was from Jackson Immunoresearch and anti-mouse-FITC secondary antibody is from Beckman Coulter (IM1619). Biotin anti-mouse CD47 antibody (miap301) and APC/Cy7 streptavidin were purchased from Biolegend (San Diego, USA). SYTOX® AADvanced™ Dead Cell Stain Kit (S10349) was purchased from Invitrogen. LIVE/DEAD® Fixable Aqua Dead Cell Stain (L34957) is from Molecular Probes, Thermofisher Scientific. Vital nuclear dye DRAQ5 (DR50200) was purchased from Alexis Biochemicals. ATP assay (K354-100) was purchased from Biovision. Cell death (DR50200) was purchased from Alexis Biochemicals. ATP was induced with human IFN-α (IntronA, SP Europe) and 9-cis-RA (Sigma) used at 1000 U/ml and 1μmol/L, respectively. Recombinant mouse IFNα was purchased from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany). Doxorubicin was purchased from Sigma.

Synthetic peptides

A panel of 9-mer peptides was synthesized by solid-phase FMOC chemistry (Primm srl, Milan). Purity was determined by reverse-phase high-performance liquid chromatography (HPLC) and verified by MALDI-TOF analysis. Preparations of 95% pure peptides were dissolved in DMSO at a concentration of 1 mg/ml and stored at -80 °C until use. We selected 5 HLA-A*0201-restricted Cyclin D1-derived peptides (cycD122-30: LLNDRVLA, cycD1101-105: LLGATCMFV, cycD1195-202: FISNPPSMV, cycD1204-212: AAGSVVAAV, cycD1238-236: RLTTRFLSRV) and 3 HLA-B*0801-restricted Survivin-derived peptides (surv98-96: SVKKQFEEL, surv101-109: FLKLDERRA, surv127-135: TAKVRRRAI). As experimental controls, we used hTERT-derived HLA-A*0201 ILAKFLHLWL and EBV BZLF1-derived RAKFKQQL HLA-B*0801 peptides.

Apoptosis detection and western blot

Apoptosis detection was performed through the analysis of Annexin V/PI staining (Roche, 11 988 549 001). Fluorescence detection was performed with a FC500 flow cytometer (Beckman Coulter, Milan, Italy). Apoptosis was also assessed evaluating apoptotic markers by immunoblotting analysis. In particular the cleaved forms of caspase 3 and PARP were detected together with the up-regulation of NOXA (ABCAM, ab13654).

Surface detection of CRT, CD47 and HSPs

10⁶ cells/sample were collected and fixed with paraformaldehyde 0.25% in PBS for 5 minutes in ice. After being re-suspended in PBS containing 0.5% bovine serum albumin (BSA), cells were labelled with the primary antibody and incubated in ice for 30 minutes. In particular, each sample was labeled with CRT, CD47 and HSPs antibodies and the SYTOX AADvanced™ Dead Cell Stain to exclude dead cells from the analysis.

5 × 10⁴ cells/sample were acquired with ImageStreamX (Amnis, Millipore) instrument using the INSPIRE software or with Cytomix FC500 flow cytometer (Beckman Coulter).

TCL preparation and Western blot

Whole-cell lysates were obtained re-suspending 5 × 10⁶ cells in 100μl of sterile PBS and by 3 rapidly freeze-thaw cycles. Then, lysates were centrifuged at 13,000 rpm (15 minutes, 4 °C) and the recovered supernatants are quantified and diluted in PBS to 1 mg/ml and used for immunoblotting analysis and DC pulsing.

Proteins were separated by SDS-polyacrylmiade gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes. After blocking with 5% milk for 1 hour, membrane was stained with primary antibodies at 4 °C overnight and then labeled with HRP-conjugated secondary antibodies. Immunoblotting was performed using the enhanced chemiluminescence detection system (Clarity, Biorad, 1705061).

DC and CTL generation and immunophenotypic analysis

IFN-DC were obtained according to a previously described procedure. Human PBMCs collected from healthy donor’s buffy coats were isolated by Ficoll-Hypaque density gradient centrifugation.

After the purification with anti-CD14-coated micro beads (Miltenyi Biotec, 130-050-201), CD14⁺ monocytes were sorted with a magnetic device. Monocytes were cultured 3 days in 6-well plates with CellGenix™ GMP DC medium (CellGenix GmbH). 10,000 UI/ml of IFN-α and 50 ng/ml recombinant human GM-CSF (PromoKine) were added to cell culture medium to achieve DC maturation and the cells obtained were used as professional antigen presenting cells (APC).

As source of tumor antigens, lysates derived from untreated, RA/IFN-α-treated or γ-irradiated cells were obtained through 3x freeze/thaw cycles in sterile PBS. Lysates were centrifuged at 13000 rpm for 15 min at 4 °C, the supernatant recovered and quantified (Pierce™ BCA Protein Assay Kit, Thermo Scientific, #23227). 100 μg were used for loading 10⁶ IFN-DCs. Unpulsed IFN-DCs were also used as controls. For CRT blocking the specific antibody anti-CRT low endotoxin, azide free (ab211962) was added during DC pulsing.

Cytotoxic T lymphocytes were generated by co-culturing lysate-pulsed mature IFNα-DCs with autologous peripheral blood lymphocytes. In order to achieve high-specific CTLs, the cultures were weekly re-stimulated 4 times and specificity was evaluated performing cytotoxicity assays (see below).

For the immunophenotypic analyses of DCs, the cells were washed and re-suspended in PBS containing 10% rabbit serum and incubated with PE-conjugated anti-CD1 a, -CD11 c, -HLA-DR, -CD80, -CD86, -CD83 antibodies. Five days after the 1st and the 4th stimulations, CTL cultures were characterized for the amount of Treg, Th1, and Th17. All antibodies for surface markers (CD3, CD4, CD8, CD25, CD45RA, and CD127 for Treg; CD3, CD4, and CD8 for Th17) were used in an appropriate volume of 2% FCS and PBS to reduce nonspecific signal. The LIVE/DEAD® Fixable Aqua Dead Cell Stain was added to exclude dead cells from the analysis. Intraacellular FoxP3 and Ki-67 were determined using the eBioscience FoxP3 Staining Buffer Set (eBioscience) according to the manufacturer’s instructions. Briefly, after surface molecules staining, cells were fixed and permeablized with fixation/
permeabilization buffer for 30 minutes at 4 °C, washed twice, and labelled with Foxp3 and Ki-67 antibodies in the presence of permeabilization buffer at 4 °C for at least 30 minutes and, after two washes, cells were re-suspended in PBS. To evaluate IL-17, IL-22, TNF-α, and IFN-γ release, cells were pretreated with 50 ng/ml Phorbol 12-Myristate 13-Acetate (PMA, Sigma-Aldrich) and 1 μg/ml Ionomycin (Sigma-Aldrich) in the presence of Golgi-STOP solution (protein transport inhibitor containing monensin, BD Biosciences) and 10 μg/ml Brefeldin (Sigma-Aldrich) in T-cell medium for 4 h at 37°C. Cells were labelled for surface molecules, then fixed and permeabilized with the Cytofix/CytopermTM solution (BD Biosciences) for 20 minutes at 4°C, washed in PBS with 0.5% Bovine Serum Albumin (BSA; Sigma-Aldrich) and 0.1% saponin (Sigma-Aldrich) and stained with antibodies in PBS+BSA+saponin at 4°C for 20 minutes. Samples were washed twice and re-suspended in PBS for flow cytometry analysis. Cytofluorimetric analysis was performed with a Cytomics FC500 (Beckman Coulter, Fullerton, CA, USA) and a LSR-FortessaTM (Becton Dickinson) belonging to the flow cytometry core facility of our Institute, photomultiplier voltages and compensation were set with unstained and stained cells or with the CompBeads Set Anti-Mouse Ig (BD Biosciences). Flow cytometry data were analyzed with CXP (Beckman Coulter, Fullerton, CA, USA), DIVA (Becton Dickinson), and FlowJo (Tree Star, Ashland, OR, USA) software.

**HMGB1 and cytokines quantification**

Mino, DOHH2, SP53 samples were treated with RA/IFN-α and the supernatants were collected at 24, 48 and 72 hours. The quantification of HMGB1 released by cells was evaluated with ELISA kit (IBL International, ST51011) according to the protocol.

After DC differentiation with 10,000 UI/ml of IFN-α and 50 ng/ml GM-CSF for 3 days, culture medium was changed and IFN-DC pulsed with both not treated and treated cell lysates for about 16 hours and then the supernatants were evaluated for the presence of IL-10 (EH1L10), IL-1β(EH2LL1B), IL-1α (EH2IL1 A), IL-6 (EH2IL6) (Thermo Fisher Scientific), TNF-α (BMS223INSTCE) (Affimetrix eBioscience) using ELISA assay. At the time of sacrifice the serum of mice were analyzed for the presence of PGE2 (AD1-930-001, Enzo Life Sciences), IFNγ (EHIFNG, Thermo Fisher Scientific), TNFα (E-EL-H0109, Elabscience).

**Nuclear NF-kB detection**

For the analysis of NF-kB nuclear internalization, samples were fixed with 2% PFA and permeabilized with cold methanol 100%. After wash with PBS containing 0.5% BSA, cells were labeled overnight with anti-NF-kB antibody (Santa Cruz Biotechnology, sc8008) at 4°C. The day after, cells were labeled with the secondary antibody (1:50) and the vital nuclear dye DRAQ5. 5 × 10^5 cells/sample were acquired with ImageStream X (Amnis) using the INSPIRE software. The extent of internalization was calculated with an algorithm of the IDEAS software that measures the similarity score between DRAQ5 and NF-kB staining. A Similarity score is determined for every cell based on a pixel by pixel correlation of the nuclear image to the NF-kB image. The threshold of similarity score to consider translocated NF-kB was defined using as negative and positive control ML-1 cell line untreated or treated with TNF-α, respectively.

**In vitro phagocytosis assay**

Mino and DOHH2 cells were labeled with Fast™ DiA cell-labeling solution according to the protocol (Molecular Probes, D7758) and then treated or not with RA/IFNα. At the fifth day, TCLs (obtained as described previously) or cells were co-cultured with IFN-DCs at a ratio of 1:3 for 4 hours. At the end of the incubation, cells were harvested, washed and stained with CD11 c-PC5 antibody. Phagocytosis was assessed by multiparameter imaging flow cytometry that allows very accurate analysis excluding false positive events, such as cell doublets instead of a single double positive cell.

10^6 IFN-DCs were pulsed O.N. with untreated or RA/IFNα-TCLs. DCs were then incubated for 20 and 60 minutes at 37°C with DQ-OVA (Molecular Probes, D12053). Cells were then washed and labeled with CD11 c-PC5. Fluorescence was monitored by cells on Image-Stream X (Amnis). DCs incubated with DQ-OVA at 4°C were used as control.

**Cytotoxicity assay**

To determine the cytotoxic activity of tumor-specific CTL cultures standard calcein-AM release assays were performed. As targets were used Mino, DOHH2, SP53 and Granta 519 cell lines. T2-A2 cells and HLA-B*0801-restricted healthy donor-derived EBV-LCLs were also used upon a 2-hour loading with HLA-A*0201- or -B*0801-restricted peptides derived respectively from cyclin D1 and Survivin. Target cells were re-suspended in Hank’s Balanced Salt Solution (HBSS) (Sigma-Aldrich) added with 5% FCS and labeled 90 minutes at 37°C and 5% CO2 with calcein-AM (Invitrogen). The HLA-A*0201-specific mAb cr11.351 (10 mg/mL) was added to the MCL target cells and incubated at room temperature for 30 min to assess the HLA-A*0201 restriction of CTL responses. After 3 washes, target cells were seeded in V-bottom 96-wells plate, effector CTLs were added at a 10:1 effector:target (E:T) ratio and, upon 4 hours incubation at 37°C and 5% CO2, fluorescence intensity of released calcein-AM was measured in target cell’s supernatants with a SpectraFluorPlus fluorimeter (Tecan).

**RNA purification, sequencing and data analysis**

Total RNA was extracted as described previously. Indexed libraries were prepared from 1 μg/ea. purified RNA with TruSeq Stranded Total RNA Sample Prep Kit (Illumina Inc.) according to the manufacturer’s instructions. Libraries were sequenced (paired-end, 2 × 100 cycles) at a concentration of 8 pmol/L per lane on HiSeq2500 platform (Illumina Inc.).

The raw sequence reads were then aligned to the human genome (hg19 assembly) using TopHat version 2.0.10 with standard parameters. A
given mRNA was considered expressed when detected by at least ≥10 reads.

Differentially expressed mRNAs were identified using DESeq version 1.14.0.52 Firstly, gene annotation was obtained for all known genes in the human genome, as provided by Ensemble (GRCh37). Using the reads mapped to the genome, we calculated the number of reads mapping to each transcript with HTSeq-count.53 These raw read counts were then used as input to DESeq for calculation of normalized signal for each transcript in the samples, and differential expression was reported as Fold Change along with associated FDR (computed according to Benjamini-Hochberg).

Gene Ontology

Gene Ontology analysis was performed on differentially expressed mRNAs using the module Comparison Analysis of Ingenuity Pathway Analysis (IPA, QIAGEN Redwood City, www.qiagen.com/ingenuity).

Immunization of hu-PBL-NOD-scid mice

NOD SCID female mice were used at 3–4 weeks of age. Four mice for each group were injected i.p. with 30–40 × 10^6 PBL from HLA-A2 healthy blood donors, re-suspended in 0.5 ml RPMI medium. MCL lysates stocks were prepared by as described above. Four days after reconstitution, hu-PBL-NOD-scid were injected i.p. with 2 × 10^6 autologous IFN-DCs pulsed for 24 h at 37°C with TCL (200 μg). Vaccinated mice received boost immunizations at day 7th and day 14th and were sacrificed after additional 7 days.

Recovery of cells from hu-PBL-NOD-scid mice and ELISPOT assay

Hu-PBL-NOD-scid mice were sacrificed 7–10 days after last immunization. Cells were collected from the spleen. Human cells from mouse spleens were enriched by Ficoll density gradient centrifugation and pooled (three to four mice per group). Mino cells were used for stimulation of human cells recovered from hu-PBL-NOD-scid mice. PBMC cultures treated with 2 μg/ml PHA served as positive controls. CD3+ T cells were positively selected by MACS Micro Beads (Miltenyi Biotec) and tested 10^5/well in an ELISPOT assay for the production of IFN-γ and Granzyme B after incubation with Mino cells at a responder/stimulator ratio of 4:1.

Therapeutic vaccination of tumor-bearing hu-PBL-NOD-scid mice

NOD SCID mice were injected subcutaneously (s.c.) in the shoulder with 5 × 10^6 Mino cells re-suspended in 0.2 ml RPMI 1640 medium. Lymphoma cells required about 14 days to become palpable masses and hu-PBL-NOD-scid mice were reconstituted with hu-PBL from HLA-A2+ healthy blood donors before tumor masses became evident (day 9). Five mice for each group were injected i.p. with 30–40 × 10^6 PBL, re-suspended in 0.5 ml RPMI medium. The resulting hu-PBL-NOD-scid mice were vaccinated (i.p) 4 days later with 2 × 10^6 TCL-loaded IFN-DC. Mice received boost immunizations at day 7th and day 14th. Tumor growth was followed for 43 day. The two major diameters of each tumor nodule were measured by calipers and the mean tumor diameter was calculated for each tumor.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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