Improved clinical outcome in indolent B-cell lymphoma patients vaccinated with autologous tumor cells experiencing immunogenic death

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Running title: Eat-me signals in DC vaccines for indolent NHL

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ABSTRACT

Increasing evidence argues that the success of an anticancer treatment may rely upon immunoadjuvant side effects including the induction of immunogenic tumor cell death. Based on the assumption that this death mechanism is a similar prerequisite for the efficacy of an active immunotherapy using killed tumor cells, we examined a vaccination strategy employing dendritic cells (DCs) loaded with apoptotic and necrotic cell bodies derived from autologous tumors. Using this approach, clinical and immunological responses were achieved in 6/18 patients with relapsed indolent non-Hodgkin lymphoma (NHL). The present report illustrates an impaired ability of the neoplastic cells used to vaccinate non-responders to undergo immunogenic death upon exposure to a cell-death protocol based on heat-shock, γ- and UVC-rays. Interestingly, when compared to doxorubicin, this treatment increased surface translocation of calreticulin and cellular release of HMGB1 and ATP in histologically distinct NHL cell lines. In contrast, treated lymphoma cells from responders displayed higher amounts of calreticulin and HSP90 compared to those from non-responders and boosted the production of specific antibodies when loaded into DCs for vaccination. Accordingly, the extent of calreticulin and HSP90 surface-expression in the DC antigenic cargo was significantly associated with the clinical and immunological responses achieved. Our results indicate that a positive clinical impact is obtained when immunogenically killed autologous neoplastic cells are employed for the generation of a DC-based vaccine. Therapeutic improvements may thus be accomplished by circumventing the tumor-impaired ability to undergo immunogenic death and prime the antitumor immune response.
INTRODUCTION
Vaccination with cancer cells treated ex-vivo with antracyclines, or oxaliplatin, or ionizing radiation protects mice against a subsequent challenge with live tumor cells(1-4). The immunizing properties of killed tumor cells depends on the ability of a cytotoxic agent to render their death immunogenic so that the immune system can be specifically alerted to the presence of a tumor(5). Similarly, the way in which neoplastic cells are killed to produce the antigenic content of a dendritic cell (DC)-based vaccine may be crucial for priming a clinically efficacious antitumor immune response. We recently reported that vaccination with autologous monocyte-derived DCs pulsed with autologous tumor cells dying after exposure to heat shock (HS), \(\gamma\)- and UVC-rays elicited a clinical response associated with tumor-specific immune-activation in 6/18 relapsed indolent non-Hodgkin lymphoma (NHL) patients(6). We have now sought to determine whether responders (R) and non-responders (NR) can be distinguished in terms of immunogenic tumor cell killing at the time of vaccine preparation. Pre-clinical studies of radio/chemotherapy-elicited immunogenic cell death in sarcoma, breast, and colon carcinoma mouse models(1, 3, 7) have shown that the release of “eat-me” and danger signals by tumor cells is the main molecular mechanism whereby DC engulfment of dead cell particles and their activation promote the cross-priming of tumor antigens and induction of a specific adaptive immune response(8, 9). Immunogenic cell death is preceded by relocation of the endoplasmic reticulum-resident chaperone calreticulin (CRT)(1, 10, 11) to the plasma membrane, followed by surface expression of heat shock proteins (HSPs) 70 and 90(12) that act as vehicles for peptide antigens(13-15) or direct immunogenic signals for DC activation(16-19). Next, dying tumor cells release ATP (20, 21) and the nuclear factor high-mobility-group box 1 (HMGB1)(7, 22), both essential for DC activation and antigen presentation to specific cytotoxic T cells. We have assessed the ability of HS, \(\gamma\)-, and UVC-rays to induce CRT and HSP translocation as well as HMGB1 and ATP release from 3 cell lines, representing low-, intermediate-, and high-grade NHL. We then compared these outcomes to the effect of doxorubicin (DXR), a well-recognized...
immunogenic cell death inducer(1, 2, 7, 20). The results show that our cell-death protocol
was even more efficient than DXR treatment in inducing the processes that define
immunogenic cell death in all 3 NHL cell lines. As to the identification of predictors of the
clinical outcome of a cancer vaccine(6), we speculated that defective exposure of
immunogenic signals(10) by heat shocked, γ-, and UVC-irradiated tumor cells used as
antigenic cargo for a DC-based vaccine might be associated with a reduced probability of
response. When primary indolent NHL was studied, in fact, observation of a difference in the
tumor’s ability to undergo immunogenic cell death pointed to a critical association between
the amount of immunogenic signals emitted by apoptotic and necrotic tumor cell bodies and
a DC-based vaccine’s clinical efficacy.
MATERIALS AND METHODS

Cell lines, primary cells, and culture conditions. DOHH-2, SU-DHL-6, and SU-DHL-4 cell lines, representing follicular centroblastic/centrocytic, diffuse mixed small and large cell, and diffuse large cell cleaved cell NHL respectively, were purchased from DSMZ (Braunschweig, Germany) and cultured in RPMI 1640 (Lonza, Basel, Switzerland) supplemented with 10% (v/v) inactivated fetal bovine serum (Lonza), 1% (v/v) L-glutamine (Lonza) and 1% (v/v) Hepes buffer (Lonza) in a humidified chamber (95% air, 5% CO2) at 37°C. All cell lines were regularly screened to ensure absence of mycoplasma contamination by MycoAlert® Mycoplasma Detection Kit (Lonza), according to the manufacturer’s instructions. Primary lymphoma cells were isolated from lymph nodes or peripheral blood (PB) as previously reported (6). Written informed consent for the investigational use of lymphocytes, serum samples and tumor specimens was obtained from each patient. Apoptotic and necrotic cell bodies were generated by exposing primary lymphoma cells and cell lines to HS, γ-irradiation (γ), and UVC-rays (UVC) in accordance with a previously described cell death induction protocol(6, 23) referred to as ALL in this paper when compared to the same agents used singly. Lymphoma cells were also treated with DXR (Pfizer Italia s.r.l.) at the indicated doses as the positive control for immunogenic death induction.

Flow cytometry. Surface stainings were performed as described(24) after blocking nonspecific antibody (Ab) binding to the Fc-receptors (FcR blocking reagent, Miltenyi Biotec, Gladbach, Germany). The following mouse anti-human Abs were used: monoclonal FITC-labeled anti-HLA-DQ, PerCP-labeled anti-HLA-DR (BD Biosciences, San Jose, CA), PE-labeled anti-HSP70, anti-HSP90, purified anti-CRT (Stressgen, Ann Arbor, MI), and polyclonal anti-HLA class I (clone W6/32, Sera-Lab, Sussex, UK). FITC-labeled goat anti-mouse immunoglobulins (Jackson Imunoresearch, Suffork, UK) formed the secondary antibody. To avoid intracellular protein detection, dead cells were excluded by 7-Aminoactinomycin D (7-AAD; Invitrogen, Eugene, Oregon, USA) co-staining (1µg/ml for 20' at 4°C). The immunophenotypic analyses of regulatory T cells (Tregs) and natural killer cells
(NK) were determined as described (6) in pre- and post-vaccination PB samples from the patients whose immunological monitoring was not part of the previous study(6). As negative controls, cells were incubated in parallel with the proper isotype immunoglobulins (PE-labeled and purified mouse IgG1, Stressgen; FITC-labeled Rat IgG2a, eBiosciences, San Diego, CA). Samples were acquired by setting photomultiplier tubes on the autofluorescence of the related negative control. All plots were gated on high Forward Scatter (FSC) and low Side Scatter (SSC) to exclude cell debris. A minimum of 50,000-gated events was collected per sample. The Apoptosis Detection kit (Bender MedSystem, Vienna, Austria) was used in accordance with the manufacturer's protocol. Data were acquired on a BD FACSCalibur using BD CellQuest software version 3.3 (Becton Dickinson, Lincoln Park, NJ) and analyzed by FlowJo 8.7.1 software version for Macintosh (Tree Star, Inc. Ashland, OR).

**ELISA.** Cellular release of HMGB1 was measured in 24-hour culture supernatants using the appropriate ELISA kit according to the manufacturer's instructions (Shino Test Corporation, Kanagawa, Japan).

**ATP release assay.** Extracellular ATP was measured in 24-hour culture supernatants by means of the luciferin-based ENLITEN ATP Assay (Promega, Madison, WI). Light emission was recorded with a Berthold luminometer (Berthold Detection systems GmbH, Pfortzheim, Germany).

**Purification and biotinylation of human immunoglobulins.** Ab purification from human serum and immunoglobulin biotinylation were performed as described(6).

**Western Blot.** DOHH-2 cells or apoptotic and necrotic cell bodies were lysed for 1 hour on ice in lysis buffer (50 mM Tris-HCl pH 7.2, 150 mM NaCl, 100mM NaF, 100mM sodium pyruvate, 1% Triton X-100) containing protease inhibitors, 2 mM phenylmethylsulfonylfluoride, 10 μg/ml aprotinin, and 2mM Na3VO4. Protein extracts were separated by electrophoresis on pre-cast polyacrylamide gels (Invitrogen, Carlsbad, CA), transferred to hydrophobic polyvinylidene difluoride (PVDF) membranes (Amersham, Pittsburgh, PA), probed with biotinylated human immunoglobulins, anti-CRT, anti-HSP90 and anti-HSP70 (Stressgen) monoclonal Abs or rabbit anti-human actin polyclonal Ab.
(Sigma, Milan, Italy), and visualized as previously described(25). Full-range Rainbow molecular marker (12-225 kDa, Amersham, GE Healthcare) and Sharp Protein Standard (3.5-260 kDa, Novex, Invitrogen) were run in parallel in each SDS-PAGE analyses.

**In-gel tryptic digestion, MALDI-TOF-MS analysis, and peptide mass fingerprinting.** For protein profiling of DOHH-2 apoptotic and necrotic cell bodies, protein bands were excised from Coomassie-stained preparative gels and processed as previously described(26). Matrix-assisted laser desorption/ionization, time-of-flight mass spectrometry (MALDI-TOF-MS) was carried out with a Voyager-DE STR (Applied Biosystems; Milan, Italy), equipped with a nitrogen laser (337 nm). Monoisotopic peptide masses were analyzed using Aldente software (http://www.expasy.ch/tools/aldente/). Input was searched according to the following database: Aldente, UniProtKB/SwissProt; predefined taxon: Mammalia; Spectrometer internal error max: 25. Only proteins identified from 3 or more separate experiments were considered.

**Gene Ontology analysis.** The Database for Annotation, Visualization, and Integrated Discovery (DAVID; http://www.david.niaid.nih.gov) was used to discover the gene ontology biological processes, cellular components, molecular functions (GO terms), and KEGG pathways significantly represented by proteins from DOHH-2 apoptotic and necrotic bodies.

**Statistical analysis.** Statistical significance was calculated using the 2-sided Student “t” test (p ≤0.05). Pearson and Spearman correlation coefficients were calculated to measure dependence between variables. Statistical analyses were performed on the Prism 5.0a software version for Macintosh (GraphPad Software, Inc.).
RESULTS

Immunogenic signals in heat shocked, γ-, and UVC- irradiated NHL cell lines

The extent of CRT cell surface translocation, HMGB1, and ATP release were evaluated in DOHH-2, SU-DHL-6, and SU-DHL-4 cell lines treated with HS, γ, UVC, or ALL. Cells were treated with DXR in parallel as positive control. The extent of CRT cell surface translocation was directly proportional to the amount of DXR in a limited dose interval (0.1-20 µM), and peaked at 0.5, 2.5, and 15 µM for DOHH-2, SU-DHL-6, and SU-DHL-4, respectively (data not shown). DXR was therefore administered at 0.5, 2.5, and 15 µM in cultures of DOHH-2, SU-DHL-6, and SU-DHL-4 cells respectively, and approximately 50% cell growth inhibition was reached after 24 hours (Figure 1Ai). ALL was highly cytotoxic against the 3 NHL cell lines tested, as shown by the trypan blue exclusion test and flow cytometry analysis of apoptosis (Figure 1Ai and Supplemental Figure 1A). ALL-treated cells expressed surface-CRT at a higher frequency (Figure 1Aii, white columns, and Supplemental Figure 2A for a representative example) and intensity (Figure 1Aii, gray columns, and Supplemental Figure 2A for a representative example) compared to cells exposed to γ, UVC or DXR alone. ALL provided a similar amount of CRT cell-surface translocation, regardless of lymphoma histological grade (Figure 1Aii, left vs. middle vs. right panel, and Supplemental Figure 2A for a representative example). As additional signals promoting the recognition and uptake of dying cells by DCs, the surface-expression of stress-inducible HSP90 and HSP70 was evaluated. ALL significantly enhanced that of HSP90 in DOHH-2, SU-DHL-6, or SU-DHL-4 compared to γ or UVC alone (Figure 1Aiii and Supplemental Figure 2B for a representative example), but that of HSP70 only in SU-DHL-6 cells and to a lesser extent (Supplemental Figure 1B and Supplemental Figure 2C for a representative example). The ability of DXR and ALL to stimulate cellular release of HMGB1 and ATP, two distinctive signals of immunogenic cell death, was also compared. For each cell line tested, ALL provided a significantly higher release of HMGB1 and ATP from each cell line than DXR administration as revealed by specific ELISA and luciferin-based assays in 24-hour conditioned culture.
media (Figure 1Bi, p=0.045, p=0.029, p=0.0002; Figure 1Bii, p=0.049, p=0.045, p=0.024).

Interestingly, CRT and HSP90 exposure in heat-shocked NHL cell lines was comparable to that achieved in ALL treated cells (Figure 1Aii-iii); however, HS alone was less efficient than ALL to boost ATP and HMGB1 release from all 3 cell lines (data not shown). Combination of $\gamma$ and UVC with HS was thus a harmful way of inducing all reported mediators of immunogenic cell death in the 3 differently aggressive NHL cell lines. Coomassie staining revealed that ALL deeply modified the DOHH-2 cell protein pattern (Supplemental Figure 3A, B). MS-based multiplexed profiling of apoptotic/necrotic DOHH-2 cell bodies highlighted an enrichment in proteins involved in mitochondrion and endoplasmic reticulum functions, ATP metabolism, intracellular organelle reorganization, and stress response, which are represented in 15 gene ontology (GO) categories (Table 1 and Supplemental Table 1).

Seven KEGG pathways, including oxidative phosphorylation, citrate cycle, and antigen processing and presentation were also significantly represented in ALL-treated DOHH-2 cells (Table 2). Most of the functions and pathways detected were integrally involved in apoptosis and in biochemical events associated with the immunogenicity of dying cells, and thus emphasized the ability of HS associated with $\gamma$ and UVC to generate immunogenic apoptotic and necrotic bodies from NHL cell lines.

**Immunogenic cell-death in primary indolent NHL exposed to heat shock, $\gamma$- and UVC-rays**

The results obtained in long-term NHL cell lines prompted us to investigate whether combined exposure to HS, $\gamma$, and UVC was the best way to induce immunogenic cell death in primary B-cell NHL. Since in our previous study(6) ALL constituted the cell death protocol adopted to kill indolent NHL primary cells and obtain tumor antigen cargo for patient-specific anti-lymphoma DC-based vaccines, we analyzed the surface exposure of CRT and HSPs and the release of HMGB1 in treated follicular lymphoma (FL) cells isolated from 4 patients (Table 3, patients #1, #10, #11, #14) prior to immunotherapy. After 24 hours, ALL killed over 90% of tumor cells as revealed by flow cytometry analysis of apoptosis and reached...
statistical significance when compared to UVC or HS (Supplemental Figure 4A; p=0.049, 
p=0.047). However, for each treatment condition no significant differences where found 
between tumor cells from R and NR in terms of amount of cell death (Supplemental Figure 
4B). Since only a trend toward increase of CRT and HSP90 surface expression was 
observed in ALL as compared to single agent-treated tumors (average of 4 FL samples, data 
not shown), the ability of dying FL cells to determine these parameters was assessed in 
samples from R (Table 3, patients #1, #14) and from NR (Table 3, patients #10, #11), 
separated into 2 groups. The extent of CRT exposure on ALL treated tumor cells from R was 
significantly higher compared to the same samples exposed to single agents, as revealed by 
flow cytometry analysis of the median fluorescence intensity (MFI; Figure 2A, white columns; 
p=0.022, p=0.037, p=0.024). ALL significantly increased CRT and HSP90 surface 
expression in tumor cells from R as compared to NR (Figure 2A, B white vs. gray columns, 
p=0.006, p=0.045; Supplemental Figure 5A, B). No statistically significant differences were 
found when HSP70 exposure or HMGB1 release were analyzed (data not shown). These 
results indicate that equally treated primary FL, with a similar clinical history, may differ in 
their ability to emit immunogenic signals. To determine whether the immunization efficacy of 
a DC-based vaccine depends on the extent of immunogenic signal exposure by dying tumor 
cells loaded on DCs, we searched for anti-CRT and -HSP90 Abs in pre- and post- 
vaccination serum samples. Apoptotic and necrotic DOHH-2 cells were used as target cells 
in western blot analyses, since upon exposure to ALL they displayed surface CRT and 
HSP90 (Figure 1Aii, Aiii) as well as a significant proteome enrichment of HSP90 and Erp57, 
the cognate functional partner of CRT(27) (Supplemental Table 1). Protein extracts were 
probed with biotin-conjugated immunoglobulins purified from pre- and post–vaccination 
serum samples of R (Figure 3Ai, ii, Figure 3Bi, ii and Supplemental Figure 6) and NR (Figure 
3Aiii, iv, Figure 3Biii, iv and Supplemental Figure 6) or commercial mouse monoclonal anti- 
CRT (Figure 3Av and Supplemental Figure 6) and anti-HSP90 (Figure 3Bv and 
Supplemental Figure 6) Abs. Remarkably, after vaccination clinical R showed a greater 
amount of circulating Abs directed against proteins migrating at molecular weights
compatible with CRT (Figure 3Ai, ii vs. 3Av) and HSP90 (3Bi, ii vs. 3Bv) compared to NR (Figure 3Aiii, iv vs. 3Av and Figure 3Biii, iv vs. 3Bv), and thus provided evidence for a positive association between tumor cell ability to expose immunogenic signals and their immunizing properties when loaded into DCs and injected into patients.

**Immunogenic tumor cell death and clinical efficacy of vaccination with killed autologous NHL cell-pulsed DCs**

To determine the involvement of immunogenic tumor cell death in the efficacy of DC-based active immunotherapy in indolent NHL patients, the ability of tumor cells to provide immunogenic signals upon exposure to ALL was studied in all samples still available from vaccinated patients (R=6; NR=8) according to their outcome after vaccination (Table 3). While the extent of cell death did not differ between the two groups (Figure 4A, p=0.127), CRT positive cell percentage and MFI were significantly higher in dying tumor cells from R compared to NR, as revealed by flow cytometry (Figure 4B, left upper panel p=0.023 and left lower panel p=0.039). Furthermore, apoptotic and necrotic bodies from tumor cells of R expressed surface HSP90 at a higher frequency and intensity compared to those from NR (Figure 4B, middle upper panel, p=0.001 and middle lower panel, p=0.002); no significant differences were found when HSP70 was analyzed (Figure 4B, right upper panel p=0.421 and right lower panel p=0.481). Flow cytometry analyses of HLA class I and II expression on dying tumor cells from R and NR revealed no significant differences (data not shown), suggesting that they shared similar antigen presentation properties. Similarly, HMGB1 release from dying tumor cells did not differ between R and NR (Figure 4C, p=0.705). We then measured the strength of the correlation between the extent of CRT and HSP90 exposure on autologous killed tumor cells used to load DCs for vaccine preparation and patient outcome after vaccination. We found a significant positive association between HSP90 surface-expression in dying tumor cells and the occurrence of clinical responses (HSP90 MFI, Spearman r=0.8631, p<0.0001; %HSP90\(^+\) cells, Spearman r=0.8235, p=0.0003). The frequency of tumor cells expressing surface-CRT also significantly correlated with a favorable outcome after vaccination (Spearman r=0.6087; p=0.0209).
previously reported(6), R showed tumor-specific T and B cell activation, Treg frequency reduction and NK cell maturation, indicating a potential association between the exposure of immunogenic signals and the ability of a DC-based vaccine to induce a clinically efficient immune activation. Accordingly, clinical responses were significantly associated with NK cell maturation (Spearman r=0.7835, p=0.0015) and Treg frequency reduction (Spearman r=-0.7835, p=0.0015), which, in turn, were found to directly correlate with the extent of CRT and HSP90 expression in the vaccine antigenic cargo (CRT MFI vs. NK maturation: Pearson r=0.8321, p=0.0004; %CRT+ cells vs. NK maturation: Pearson r=0.7506, p=0.0031; CRT MFI vs. Treg frequency reduction: Pearson r=-0.6594, p=0.0142; HSP90 MFI vs. Treg frequency reduction: Pearson r=-0.6164, p=0.0249; %HSP90+ cells vs. Treg frequency reduction: Pearson r=-0.6716, p=0.0119).
DISCUSSION

Our study confirms in a human setting recent findings obtained in mice concerning the possibility of inducing immunogenic death in cancer cells in order to provide a reliable source of antigens for antitumor vaccination. We found that when 3 human NHL cell lines representing low-, intermediate- and high-grade lymphomas were treated with HS, γ, and UVC they displayed all of the key features required to trigger a DC-mediated antitumor immune response, including the plasma membrane translocation of CRT and HSPs, and the release of HMGB1 and ATP. Since these events were enhanced when the three agents were co-administered, it is necessary to combine HS, γ, and UVC to efficiently induce an immunogenic tumor cell death. This combination was also more effective than DXR at inducing surface translocation of “eat-me” signals coupled with the release of HMGB1 and ATP. Apoptotic and necrotic tumor cell bodies generated through the exposure of primary indolent NHL cells to HS, γ, and UVC were used to pulse autologous monocyte-derived DCs and thus produce a patient-tailored vaccine. Interestingly, while displaying the same level of apoptosis, necrosis, and HMGB1 release, primary lymphoma samples from R were better able to translocate CRT and HSP90 to the cell surface upon exposure to the combined treatment as compared to NR. Remarkably, vaccination with DCs pulsed with ALL-treated autologous tumors induced the production of circulating anti-CRT and anti-HSP90 Abs in R, but not in NR, and elicited clinical responses strongly associated with multifaceted antitumor immune activation. These observations in an admittedly small number of cases point to a positive correlation between the surface-expression of CRT or HSP90 in dying tumor cells used as antigen cargo for a DC-based vaccine and immunological responses associated with clinical benefit.

Our results are in line with Zitvogel et al.’s findings that failure of one early step towards immunogenic cancer cell death was sufficient to abrogate the process. The lack of the CRT exposure alone, for example, may explain the hampered ability of cisplatin to induce immunogenic death in mouse colon cancer cells as compared to oxaliplatin, and thus reflect...
its limited therapeutic efficacy in colon carcinoma patients(3). Conversely, in our setting the same treatment resulted in a different exposure of “eat-me” signals in clinically comparable primary indolent NHL, while reproducibly providing human NHL cell lines of different histologic grades with these immunogenic molecules. Similarly, HS exposure alone was sufficient to kill most of the cells in the 3 NHL cell line cultures and boost their translocation of CRT and HSP90, whereas it was less capable of eliciting the same effects in primary tumors. Alterations in the molecular pathways for chaperone trafficking(10), which render tumor cell death immunogenically silent, may confer survival advantages to B-cell NHL in vivo and may be selected as new immune-escape mechanisms. Therefore primary tumor cells may be less prone to undergo immunogenic cell death compared to in vitro established cell lines. Accordingly, the ability to down-regulate CRT expression has been shown to be associated with a negative prognostic/predictive impact in colon cancer(28), neuroblastoma(29), and cervical carcinoma(30), as well as in follicular thyroid carcinoma(31). Nevertheless, our in vitro models consistently demonstrated CRT and HSP90 cell surface translocation are the distinctive features induced by the combined exposure to HS, γ, and UVC. CRT’s involvement in immunogenic tumor cell death is also illustrated by its long investigation on account of its immunostimulatory properties, including its ability to elicit a CTL response against chaperoned antigens(13) or its own antigenic epitopes(32) as well as to induce specific auto-antibodies in a variety of autoimmune diseases(33). HSP expression on the surface of dying tumor cells similarly improves their recognition by DCs(12), and increases the efficient cross-presentation of tumor-derived chaperoned antigenic peptides(14) and DC maturation(17, 34). Chaperone rich tumor cell lysates activate NK cell effector functions in the presence of accessory cells such as DCs(35). Chaperone proteins may thus be endowed with a key role in the cross-talk between DCs and NK cells(36). By killing tumor cells, activated NK cells may render tumor antigens available for further DC cross-presentation in vivo(37), while inhibiting peripheral Treg conversion and directly lysing Tregs(38, 39). Regression of the tumor mass may thus result in re-establishment of the balance between immunity and tolerance in favor of tumor
Eat-me signals in DC vaccines for indolent NHL

1 immune surveillance(40). A favorable clinical outcome after vaccination, in fact, was found to
2 be significantly associated with the extent of chaperone protein expression on apoptotic and
3 necrotic tumor cell bodies loaded into DCs, and, in turn, with NK cell activation and Treg
4 frequency reduction(6). In keeping with the extensively described property of immunogenic
5 tumor cell death to elicit specific T cell responses(1, 8, 41), antitumor adaptive cellular
6 immunity was detected at tumor site in partial R and in PB in one complete R, for whom a
7 tumor-specific idiotype T cell response was assessed(6).
8 All our R showed an increased ability to translocate HSP90 as compared to NR, whereas 3
9 R displayed comparable ability in CRT exposure with respect to NR, meaning that at least
10 one “eat-me” signal was sufficient to provide the proper stimuli for DC antigen uptake and
11 activation. The considerable expression of HSP90 in vaccines administered in all R may
12 have compensated for the limited release of CRT in 3 of them.
13 These results delineate new ways of optimizing anticancer vaccines for the stimulation of a
14 therapeutic antineoplastic immune response. Pulsing of DCs ex-vivo with immunologically
15 killed tumor cells avoids their physiological clearance by neighboring cells before entering
16 the late stages of the apoptotic process(19), and thus ensures optimal DC antigen-uptake
17 and activation. An explanation may thus be found for the advantages gained by using
18 apoptotic and necrotic tumor cells to generate specific DC vaccines(42). In addition,
19 characterization of the molecular mechanisms responsible for cell death immunogenicity
20 may provide novel strategies to favor its occurrence during the preparation of killed tumor-
21 cell-based vaccines. Our results, indeed, showed that dying neoplastic cells, due to their
22 impaired ability to expose CRT or HSP90, lose their immunogenic properties. Since Bcl-2
23 can impair CRT surface translocation(10) and its up-regulation constitutes one of the
24 hallmarks of indolent NHL, in particular FL, we investigated whether in our series Bcl-2 over-
25 expression could explain the reduced tumor cell ability to expose CRT. However, we did not
26 find a precise correspondence between these two features (data not shown), and the almost
27 complete consumption of tumor cells samples for most of our vaccinated patients hampered
28 further investigations of the mechanisms that account for these deficiencies. As
demonstrated for murine colon carcinoma and sarcoma models(11), cytotoxic agents can be combined with adjuvant compounds such as recombinant CRT or PP1/GADD34 inhibitors to enhance CRT surface expression, oligodeoxynucleotides (i.e., class A CpG), and cytokines (i.e., IL-1β) in order to boost DC engulfing, cross-presentation, and maturation, and new ways of increasing the potency of DC-based vaccination may thus be devised(9, 43).
References


Table 1: Gene Ontology (GO) of proteins significantly represented in DOHH-2 apoptotic and necrotic bodies.

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<thead>
<tr>
<th>GO Terms</th>
<th>Fold Enrichment</th>
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<tr>
<td>Endoplasmic reticulum</td>
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<td>Actin cytoskeleton organization</td>
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<td>Heat-shock proteins (HSP70)</td>
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<td>ATP carrier protein</td>
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Table 2: KEGG pathways significantly represented by ALL-treated DOHH-2 cells.

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<th>Fold Enrichment</th>
<th>p</th>
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<td>hsa04810: Regulation of actin cytoskeleton</td>
<td>10.53</td>
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<td>hsa00190: Oxidative phosphorylation</td>
<td>12.28</td>
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<td>hsa04612: Antigen processing and presentation</td>
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<tr>
<td>hsa00010: Glycolysis/Gluconeogenesis</td>
<td>7.02</td>
<td>6.9</td>
<td>1.87E-002</td>
</tr>
<tr>
<td>hsa05040: Huntington’s disease</td>
<td>5.26</td>
<td>10.6</td>
<td>3.04E-002</td>
</tr>
<tr>
<td>hsa00710: Carbon fixation</td>
<td>5.26</td>
<td>13.4</td>
<td>1.96E-002</td>
</tr>
<tr>
<td>hsa00020: Citrate cycle (TCA cycle)</td>
<td>7.02</td>
<td>13.7</td>
<td>2.69E-003</td>
</tr>
</tbody>
</table>
Table 3: Clinical and immunological characteristics of vaccinated patients.

<table>
<thead>
<tr>
<th>UPN #</th>
<th>Age/Sex</th>
<th>NHL Type, Stage</th>
<th>Previous Treatment (Response/Duration)</th>
<th>Clinical Response (Length in Months)</th>
<th>Immunological Responses</th>
<th>Immunogenic Signals in Dying Tumor Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Post/Pre Vax Activated NK Cell Frequency***</td>
<td>Post/Pre Vax Antitumor T Cells**</td>
</tr>
<tr>
<td>12</td>
<td>65/M</td>
<td>LP, Stage IA</td>
<td>6 CVP (CR/36 months)</td>
<td>CR (67)</td>
<td>1.08</td>
<td>0.82</td>
</tr>
<tr>
<td>13</td>
<td>72/F</td>
<td>FL grade I, Stage IIA</td>
<td>6 CVP (PR/16 months); 4 Rituximab (CR/24 months)</td>
<td>CR (64)</td>
<td>1.37</td>
<td>n.a.</td>
</tr>
<tr>
<td>14</td>
<td>52/F</td>
<td>FL grade IIIa, Stage IVA</td>
<td>6 R-MegaCEOP (PR/10 months); HDS (CR/6 months)</td>
<td>CR (63)</td>
<td>2.64</td>
<td>15*</td>
</tr>
<tr>
<td>1</td>
<td>49/F</td>
<td>LP, Stage IVA</td>
<td>6 CVP (CR/48 months); 8 Rituximab (PR/72 months)</td>
<td>PR (47)</td>
<td>0.99</td>
<td>3.02</td>
</tr>
<tr>
<td>5</td>
<td>52/M</td>
<td>FL grade I, Stage IVA</td>
<td>6 CVP (CR/24 months); 4 Rituximab (PR/15 months)</td>
<td>PR (12)</td>
<td>1.11</td>
<td>4.84</td>
</tr>
<tr>
<td>6</td>
<td>45/M</td>
<td>FL grade II, Stage IVA</td>
<td>6 R-CEOP (CR/12 months); 4 Rituximab (PR/8 months); HDS (CR/12 months)</td>
<td>PR (7)</td>
<td>1.14</td>
<td>4.75</td>
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<tr>
<td>4</td>
<td>51/M</td>
<td>FL grade II, Stage IA</td>
<td>4 Rituximab (CR/24 months); RT 30 Gy (CR/24 months)</td>
<td>SD (78)</td>
<td>n.a.</td>
<td>1.21</td>
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<tr>
<td>9</td>
<td>63/M</td>
<td>LP, Stage IVA</td>
<td>8 Rituximab (PR/16 months); 4 CVP (PR/18 months); 4 R-CHOP (SD/32 months)</td>
<td>SD (69)</td>
<td>0.62</td>
<td>1.01</td>
</tr>
<tr>
<td>10</td>
<td>54/M</td>
<td>FL grade I, Stage IVA</td>
<td>8 R-CVP (CR/36 months)</td>
<td>SD (68)</td>
<td>1.06</td>
<td>1.13</td>
</tr>
<tr>
<td>11</td>
<td>72/M</td>
<td>FL grade II, Stage IVB</td>
<td>18 mo Leukeran (CR/84 months)</td>
<td>SD (10)</td>
<td>0.57</td>
<td>0.89</td>
</tr>
</tbody>
</table>
### Eat-me signals in DC vaccines for indolent NHL

<p>| | | | | | | |</p>
<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>18</td>
<td>62/F</td>
<td>FL grade I, Stage IIIA</td>
<td>6 R-CVP (CR/36 months); 4 Rituximab (CR/26 months)</td>
<td>SD (52)</td>
<td>1.00</td>
<td>0.98</td>
</tr>
<tr>
<td>7</td>
<td>50/M</td>
<td>FL grade I, Stage IVA</td>
<td>6 CHOP (PR/12 months); 4 Rituximab (PR/6 months)</td>
<td>PD</td>
<td>0.86</td>
<td>1.05</td>
</tr>
<tr>
<td>8</td>
<td>56/M</td>
<td>FL grade II, Stage IA</td>
<td>3 CHOP-bleo/3 CVP (RC/16 months); HDS (CR/24 months); 8 Rituximab (CR/6 months)</td>
<td>PD</td>
<td>0.76</td>
<td>0.85</td>
</tr>
<tr>
<td>15</td>
<td>72/M</td>
<td>LP, Stage IA</td>
<td>3 CHOP (PR/8 months); Splenectomy + RT (PR/12 months); 12 months Leukeran (PR/7 months); HDS (CR/12 months); 4 Rituximab (PD/n.a.)</td>
<td>PD</td>
<td>0.84</td>
<td>0.91</td>
</tr>
</tbody>
</table>
Eat-me signals in DC vaccines for indolent NHL

UPN: Unique Progressive Number; LP: lymphoplasmocitoid lymphoma; CR: complete remission(44); PR: partial response(44); SD: stable disease(44); PD: progressive disease(44); CVP: cyclophosphamide, vincristine and prednisone; R-CVP: Rituximab plus CVP; CHOP: cyclophosphamide, adryamicin, vincristine and prednisone; R-CHOP: Rituximab plus CHOP; RT: Radiotherapy; CEOP: cyclophosphamide, epiadryamicin, vincristine and prednisone; R-CEOP: Rituximab plus CEOP; HDS: High dose sequential chemotherapy; autoBMT: autologous bone marrow transplantation; alloBMT: allogeneic bone marrow transplantation; LN: Lymph-nodes; BM: Bone marrow; PB: Peripheral blood; tx Therapy; n.a.: not assessable.

II: Post/pre vaccination ratio of activated NK cell frequency measured by FACS analysis of CD16 expression in CD3−CD56dim-gated cells(6).

**: Post/pre vaccination ratio of antitumor T cell frequency at tumor site measured by IFN-γ ELISPOT assay(6).

* : Post/pre vaccination ratio of idiotype-specific T cell frequency in PB measured by IFN-γ ELISPOT assay(6).

§ : Post/pre vaccination ratio of Treg frequency measured by FACS analysis of CD25+FOXP3+ in CD3+CD4+-gated T cells(6).
Figure Legends

Figure 1: Immunogenic cell death in NHL cell lines. Twenty-four hours following exposure to DXR or UVC- (UVC), γ-irradiation (γ) and heat shock (HS) as single or associated (ALL) treatments, the ability of NHL cell lines to undergo immunogenic death was assessed. Non-treated cultures (NT) were included as control. (A) Percentage of viable (white) and dead cells (gray) as determined by trypan blue exclusion test (i); flow cytometry analyses of CRT (ii) and HSP90 (iii) relative median fluorescence intensity (MFI, gray columns) and positive cell frequency (white columns). Relative MFI was calculated as the ratio between stained sample and negative control MFI. Analyses were performed on 7-AAD negative-gated events. Left, middle, and right subpanels indicate the results obtained with DOHH-2, SU-DHL-6, and SU-DHL-4 cell lines, respectively. (B) Quantification of HMGB1 (i) and ATP (ii) in culture supernatant. For each cell line, ATP release was normalized by the amount found in untreated cultures, giving relative ATP values. Statistically significant differences between ALL and the other culture conditions were calculated using the 2-sided Student “t” test (*p ≤ .05; **p ≤ .001; ***p ≤ .0001). The average values of results obtained in 3 independent experiments are reported.

Figure 2: CRT and HSP90 expression on treated tumor cells from vaccinated patients. Flow cytometry analyses of CRT (A) and HSP90 (B) relative MFI in tumor cells from 2 R (average patients #1 and #14, white columns) and 2 NR (average patients #10 and #11, gray columns), 24 hours after exposure to UVC-rays (UVC), heat shock (HS) and γ-irradiation (γ) as single or associated treatments (ALL). Relative MFI was calculated as the ratio between stained sample and negative control MFI of high FSC and low SSC-gated events. Significance was calculated using the 2-sided Student “t” test (*p ≤ .05; **p ≤ .001).

Figure 3: Vaccine-specific humoral response in clinical R. Western blot analyses of serum-derived immunoglobulin reactivity on DOHH-2 apoptotic and necrotic body extracts. (A) Reactive bands obtained by probing with pre- (lane 1, upper) or post- (lane 2, upper) vaccine immunoglobulins from 2 R (i, patient #1; ii, patient #14) and 2 NR (iii, patient #10; iv,
patient #11) or with anti-CRT commercial monoclonal Ab (v, upper). (B) Reactive bands obtained by probing with pre- (lane 1, upper) or post- (lane 2, upper) vaccine immunoglobulins from R (i, patient #1; ii, patient #14) and NR (iii, patient #10; iv, patient #11), or with anti-HSP90 commercial monoclonal Ab (v, upper). Actin protein expression is reported at the bottom of each panel as loading control. Arrows indicate specific bands for CRT and HSP90. Images were acquired on ArtixScan F1 scanner (Microtek International Inc., Hsin-Chu, Taiwan) using SilverFast Launcher software (MicrotekSDK) and processed with Photoshop CS4 software (Adobe Systems Incorporated, San Jose, CA).

**Figure 4: Lethal and immunogenic response in tumor cells from R and NR.** Twenty-four hours following exposure to ALL, the ability of tumor cells from R and NR to undergo immunogenic death was assessed. Flow cytometry analyses of (A) propidium iodide* tumor cell frequency (% PI+ cells); (B) positive cell frequency (upper) and MFI (lower) for CRT (left), HSP90 (middle) and HSP70 expression (right); and (C) quantification of HMGB1 release in culture supernatant from apoptotic and necrotic tumor cells. Significance was calculated using the 2-sided Student “t” test (ns= non significant difference; *p ≤ .001; **p ≤ .001; ***p ≤ .0001).
Figure 1

A

1. Viable/dead cells (%)

- DOHH-2
- SU-DHL-6
- SU-DHL-4

2. CRT expression

3. HSP90 expression

B

1. HMGB1 (ng/ml)

- ALL
- DXR

2. Relative ATP
Figure 2

(A) CRT Relative MFI

(B) HSP90 Relative MFI

(R) and NR conditions are compared across different treatments: UVC, HS, γ, and ALL. * and ** indicate statistical significance.
Figure 4
Cancer Research

Improved clinical outcome in indolent B-cell lymphoma patients vaccinated with autologous tumor cells experiencing immunogenic death

Roberta Zappasodi, Serenella M Pupa, Gaia C. Ghedini, et al.

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