

Whole lymphoma B cells allow efficient cross-presentation of antigens by dendritic cells.

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Running title

Cross-presentation of tumor antigen in lymphoma

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Abstract

Background

In order to compensate the paucity of defined tumor antigens in non-Hodgkin lymphomas, a promising approach might be the use of whole tumor cells as source of tumor antigens to pulse antigen-presenting cells. However, it is not presently known how the tumor cells should be delivered to antigen presenting cells to optimize the cross-presentation of tumor antigens to anti-tumor CD8 T cells. Here we aimed at comparing CD20-opsonized, apoptotic, and necrotic human tumor cells for their capacity to induce endocytosis and cross-presentation of tumor-associated antigens by dendritic cells or macrophages.

Methods

Endocytosis of human tumor-derived material by macrophages or dendritic cells was monitored by flow cytometry. We used the previously described influenza model and studied cross-presentation of viral antigens as cellular surrogate tumor-associated antigens by antigen-presenting cells after endocytosis of lymphoma B cells treated by inactivated influenza virus.

Results

Optimal endocytosis was obtained when tumor cells were opsonized by an anti-CD20 antibody, and as expected, macrophages were more phagocytic than dendritic cells. However, antigens from opsonized, apoptotic, and live cells but not from necrotic lymphoma cells were efficiently cross-presented by dendritic cells, but not by macrophages.

Discussion

We developed a new model with human primary lymphoma cells to study the cross-presentation of tumor-associated antigens by APCs. The results we obtained support the use of whole lymphoma cells from patient to pulse dendritic cells to induce antitumor immune response.

Keywords

Macrophages, Dendritic cells, Antigen Presentation, Lymphoma

Introduction

Despite improved clinical strategies for the treatment of non-Hodgkin's lymphomas, relapse rate still remains elevated. The advent of monoclonal antibodies, rituximab in particular, has revolutionized the clinical prognosis of low-grade lymphomas, leading to clinical responses in a substantial proportion of patients, and adds to the armament of conventional chemo- and radiotherapy. However, disease relapses occur because of acquired resistance to the original and ensuing treatments. There is a need for new strategies, and cellular immunotherapy could provide such a complementary approach by recruiting effectors of the adaptive immune system against tumors. Although a growing number of tumor-associated antigens has been discovered in the last decade, particularly in melanoma, very few tumor antigens are known in non-Hodgkin's lymphomas. The tumor Ig idiotype has already been used successfully in clinical trials [1, 2]. The reverse immunology methodology has defined the catalytic subunit of telomerase as a potential shared tumor antigen [3], but its immunological significance has still to be examined in a vaccination setting. Therefore, an alternative strategy in these malignancies is the use of the complete set of unknown tumor antigens, in the form of whole tumor cells. A critical parameter in this case is the capacity of the vaccine to induce cross-presentation of tumor antigens by antigen presenting cells (APC), i.e. presentation of internalized antigens in association with MHC class I molecules. In some murine models, cell-associated antigens are cross-presented with much higher efficiency *in vivo* and *in vitro* than soluble antigens [4-6]. Efficient uptake of tumor-derived material by endocytosis is likely to be important. Macrophages, and particularly immature dendritic cells can take up antigenic material from live or dead cells [7, 8], either apoptotic or necrotic. It has also been shown that antigens associated with immune complexes [9, 10] or opsonized cells [11, 12] are efficiently cross-presented by dendritic cells and can be used to generate anti-tumor cytotoxic T lymphocytes, *in vitro* [12] and *in vivo* [13].

In this paper, we sought to compare the different forms of human lymphoma cells for their capacity to induce cross-presentation of cellular antigens by macrophages or dendritic cells in order to determine the best way to use whole tumor cells in a cell therapeutic approach. We evaluated in particular the use of rituximab to target tumor cells toward antigen presenting cells.

Material and methods

Cells

Lymphoma B cells were prepared from invaded spleens of patients with follicular lymphoma (n=1), mantle cell lymphoma (n=2) or small lymphocytic lymphoma (n=1), containing more than 95% tumor cells, as assessed by CD19 and Ig light chain staining[14]. All patients gave informed consent to participate to this study. Malignant cells were cryopreserved in liquid nitrogen and thawed immediately before use. Viability after thawing was higher than 85%, as measured by Annexin V and Propidium iodide staining.

Peripheral blood was obtained from HLA-A2+ healthy volunteers who gave informed consent. Peripheral blood mononuclear cells were isolated by density-gradient centrifugation. Monocytes were purified with Rosette Sep isolation kit (Stem Cell Technologies, Meylan, France) and used to generate dendritic cells (DC) and macrophages as previously demonstrated. Dendritic cells were generated by a six-day culture in RPMI medium supplemented with 10% decompemented fetal calf serum, GM-CSF (500 U/ml, Leucomax, Schering-Plough, France) and IL-4 (10 ng/ml, Peprotech, Le Perray en Yvelines, France). Macrophages were generated in Iscove medium supplemented with 10% decompemented fetal calf serum and GM-CSF (500 U/ml), as described previously [15]. At the end of the culture, dendritic cells were 100% CD1a+, CD14- and CD83- and macrophages were 100% CD14+ and CD64+.

In order to generate anti-viral CD8+ T-cells, HLA-A2+ dendritic cells infected by influenza virus were cultured with purified autologous CD8+ T cells at a T/DC ratio of 10:1 for seven days in flat-bottom wells as previously described [16, 17]. The specificity of the antiviral CD8+ T-cells was evaluated by the production of soluble IFN γ in response to DC infected by virus.

Because peripheral blood cells from patients were not available, we used antigen-presenting cells from normal donors and tumor cells from patients in the following experiments.

Microscopy study

Lymphoma B cells were prepared as previously described [18]. They were left untreated or incubated for 30 min at 56°C to induce their necrosis, or opsonized by anti-CD20 (rituximab, 1 μ g/ml) 30 min at 4°C. For endocytosis of apoptotic cells, tumor cells were irradiated (45 Gy) and incubated for 18 hours at 37°C prior to the endocytosis assay.

B cells were cocultured at 4:1 ratio with the APC in RPMI 10% FCS for 2 hours, and endocytosis was stopped by addition of 1mmol.L⁻¹ EDTA on ice. For fluorescence microscopy experiments, APC were stained with the green dye PKH67 (Sigma, 2 μmol.L⁻¹, 20°C, 5 min), and added to PKH26-stained tumor cells. For visual counting of endocytosis, cells were cyto-centrifuged and stained with May-Grünwald-Giemsa (MGG) (Kit RAL 555, Reactifs RAL, Bordeaux, France).

Flow cytometric measurement of tumor cell uptake

Lymphoma B cells labeled with PKH26 (Sigma, 2 μmol.L⁻¹, 20°C, 5 min) were then prepared as previously described [18], to obtain apoptotic, necrotic or opsonized tumor cells. B cells were cocultured at 4:1 ratio with the APC in RPMI 10% FCS for 2 hours, and endocytosis was stopped by addition of 1mmol.L⁻¹ EDTA on ice. For identification of APC, cells were stained with FITC-conjugated anti-CD11b. In order to detect conjugates between tumor cells and APC, phycoerythrin-5 conjugated anti-CD19 and -CD20 were added to stain B cells before acquisition on a FACScan (BD). All antibodies were purchased from Immunotech (Marseille, France).

Treatment of B cells and dendritic cells by viral suspension

Lymphoma B cells or dendritic cells were thawed and incubated for 18 hours with formalin-inactivated influenza virus (strain A, New Caledonia/20/99 IVR116 (H1N1), Aventis Pasteur, Val de Reuil, France), corresponding to 137 ng/ml haemagglutinin, in RPMI 10% FCS then extensively washed. Endocytosis of virus was monitored with detection of viral haemagglutinin and nucleoprotein. Viral haemagglutinin was detected by polyclonal anti-haemagglutinin H1 rabbit Ab (bought from Virology Laboratory, Lyon, France) and PE-conjugated goat anti-rabbit. For intracellular detection of viral nucleoprotein (NP), tumor cells were centrifuged on glass slides, permeabilized with cold acetone, and stained with FITC-conjugated anti-NP antibody (Argene, France) before counter-staining with Evans blue.

Assay for cross-presentation

To demonstrate cross-presentation we used a model in which we measured the IFNγ secretion by specific anti-viral CD8⁺ T cells in response to APC loaded with tumor cells containing non-replicating influenza virus. In this assay, autologous macrophages or dendritic cells and

CD8⁺ T cells were HLA-A2⁺, whereas lymphoma B cells were HLA-A2⁻, making tumor cells unable to directly present viral antigens to CD8⁺ T cells.

On the day before cross-presentation assay, lymphoma B cells were treated with non-replicating influenza virus and then handled as described above, to obtain necrotic, apoptotic, or opsonized cells. After extensive washing, 2500 B cells were added to 5000 APC for four hours in round-bottom wells to allow endocytosis of tumor material. Then $5 \cdot 10^5$ effector T cells were added with 100ng/ml soluble CD40 ligand (soluble CD40L kit, Alexis). In preliminary experiments we found that addition of sCD40L was needed for cross-presentation, as previously described [19]. Culture supernatant was collected after 48h, and assayed for cytokine content using the Cytokine Bead Array kit (Becton Dickinson) as previously described [16].

Results

Tumor material is efficiently taken up by macrophages and dendritic cells

To measure endocytosis of tumor-derived material by APC, which may be a limiting step for cross-presentation, we used two methods: morphological examination after cyto centrifugation of cells and MGG staining (Fig. 1) and flow cytometry (Fig. 2). Lymphoma B cells were either γ -irradiated (apoptotic cells), heated (necrotic cells), opsonized by rituximab (opsonized cells), or left untreated as described [18]. By morphological examination, we observed significant endocytosis of whole tumor cells by macrophages only with rituximab-opsonized cells (Fig. 1A) whereas only marginal endocytosis of non-treated, apoptotic and necrotic cells could be observed. We did not detect any endocytosis of whole B cells by dendritic cells (Fig. 1B).

Then, to study more precisely the internalisation of tumor derived material, we measured it by flow cytometry. Indeed, the uptake of apoptotic material may involve endocytosis or macropinocytosis of shed apoptotic corpses rather than phagocytosis of whole cells [20], two internalization processes that may be undetectable by with MGG staining. The flow cytometric approach allowed us to compare the endocytosis of non-treated, apoptotic, necrotic or opsonized lymphoma cells by macrophages and dendritic cells. The principle is represented in Fig. 2A and B: tumor cells were stained with the red fluorescent dye PKH26, and added to APC. After 2 hours, cells were stained with FITC-conjugated CD11b, to discriminate APC. In order to distinguish internalization of cellular material from conjugates, B cells were stained with phycoerythrin-5-coupled CD19 and CD20. Endocytosis was assessed by the percentage of PKH26+ cells within the CD11b+(CD19/CD20)- APC (Fig 2A & Fig 2B). At +4°C no endocytosis was detected (Fig 2A). At 37°C, regardless of tumor treatment, we always found endocytosis of tumor-derived material by macrophages and dendritic cells (Fig. 2B, C and D). As expected, dendritic cells were less efficient at endocytosing than macrophages, and opsonization of tumor cells led to the highest uptake. The absence of endocytosis at 4°C indicated that it was not an artifact due to dye transfer. Consistent with a previous report [7], we found that antigen-presenting cells acquired cellular material from live untreated cells. Surprisingly, the extent of internalization of non-treated cells was in the same range as for uptake of apoptotic or necrotic cells.

Endocytosis of cellular material was confirmed by fluorescence microscopy. APC were stained with the green fluorescent dye PKH67, and after incubation with PKH26-stained B cells, internalization was visualized as colocalisation of dyes with yellow staining. As shown

in Fig. 3, extensive internalization of cellular material was seen in every case. Consistent with MGG staining, phagocytosis of opsonized cells by macrophages could be visualized by internalization of whole tumor cells, whereas in all other cases, internalized material consisted of small fragments.

We also examined maturation of dendritic cells after endocytosis of tumor cells. Whatever the treatment applied, dendritic cells did not mature, as assessed by CD83, HLA-DR, CD80 or CD86 staining (data not shown).

Dendritic cells are able to cross-present antigens from non-treated, apoptotic and opsonized but not from necrotic lymphoma cells

To evaluate whether these various treatments of lymphoma B cells promote cross-presentation, we used influenza virus as a surrogate tumor-associated antigen as previously described [8, 17].

We first verified the internalization of the virus by B cells, by detecting viral haemagglutinin in virus-treated lymphoma B cells. B cells expressed haemagglutinin at their surface after incubation with the virus at 37°C (Fig. 4A), whereas this staining was not observed at 4°C (data not shown), indicating that there was no passive adsorption of the virus onto tumor cells. Moreover, viral nucleoprotein was detected only in the cytoplasm of lymphoma cells as shown on Fig. 4B.

We then compared cross-presentation of viral antigens after endocytosis of virus-treated lymphoma cells by DC or macrophages. In two independent experiments (fig 5), DC pulsed with virus-treated tumor cells induced a higher secretion of IFN γ by CD8 T cells than virus-treated tumor cells alone, whether B cells were non-treated, apoptotic or opsonized, showing the cross-presentation of viral antigens expressed by tumor cells. By contrast, necrotic cells did not induce IFN γ secretion upon endocytosis by DC. We ruled out a spontaneous IFN γ release by T cells, as IFN γ was hardly detected in the supernatant of T cells alone or with unpulsed DC. Background IFN γ production was induced by lymphoma B cells in the absence of DC probably due to allogeneic nature of tumor cells. Indeed, as they were HLA-A2 negative, lymphoma B cells by themselves should not be able to present viral antigens to the HLA-A2⁺ specific T cells. In these two experiments, there were no striking differences between non-treated, apoptotic and opsonized cells for cross-presentation by dendritic cells. To ensure that virus was not released by B cells and endocytosed by APC, we incubated the overnight supernatants of the different cell preparations with dendritic cells and assessed T

cell response. The absence of IFN γ production by anti-influenza T cells, indicates that neither free virus nor secreted vesicles or membrane fragments were responsible for antigen transfer (data not shown). In contrast to dendritic cells, macrophages were not able to cross-present viral antigens to anti-influenza T lymphocytes, whatever the treatment of tumor cells (data not shown).

Discussion

In this paper, we aimed to determine whether whole tumor cells from lymphoma patients could represent an alternative approach to provide antigens to antigen presenting cells in the perspective of setting-up clinical immunotherapeutic trials. We previously reported that it was feasible to purify lymphoma cells from invaded biopsies[18], and to prepare functional dendritic cells from patients[15], in conditions suitable for therapy. Here, we developed an interesting model allowing to demonstrate that human whole tumor cells can be endocytosed by antigen presenting cells and then induce activation of reactive T cells, by a cross-presentation process. These results demonstrate that whole lymphoma B cells combined to antigen presenting cells could effectively induce a cross-presentation of tumor antigens and provide rationales for such an approach in the setting-up of clinical trials.

We first measured the endocytosis of tumor cells by dendritic cells. We set-up a flow cytometric method, more accurate than microscopic observation, to take into account the endocytosis of both whole tumor cells and parts of cells such as apoptotic bodies or membrane fragments. By this means, we have shown that both DC and macrophages were very efficient at endocytosing tumor-derived material, although dendritic cells were always less endocytic, as expected. Antigen transfer from lymphoma B cells to APC likely involved close cell-to-cell interactions. Indeed, incubation with supernatants of the different tumor cells did not induce any staining of APC indicating that neither shed vesicles nor cell debris were transferred to APC. In this model, the nature of the interactions and receptors involved remains to be examined except for endocytosis of opsonized cell by macrophages which involved CD32-mediated phagocytosis as described elsewhere [21]. Cellular transfer may consist in non-specific macropinocytosis or endocytosis of cytoplasmic material, or in membrane transfer from tumor cells to APC. Such a transfer has already been described with simian cells [7], and with human monocyte-derived dendritic cells [11, 22].

We next compared the repercussions of the different treatments on tumor antigen cross-presentation by APC. Because of the paucity of known tumor antigens in this pathology, we used an already described model [8, 17] and studied cross-presentation of viral antigens after treatment of allogeneic lymphoma B cells by inactivated influenza virus. As lymphoma cells are fully effective for antigen-presenting function it was not possible to set-up an autologous assay. Indeed, in a such assay, it is not possible to distinguish the IFN- γ production induced by direct antigen presentation by tumor cell and the one induced by indirect antigen presentation by dendritic cells. In our experiments, APC and anti-viral specific CD8⁺ T-cells

were autologous and expressed HLA-A2 molecules. Consequently, CD8⁺ T-cells were able to produce IFN γ only when viral antigens were cross-presented by APC. As tumor cells treated by inactivated-virus were HLA-A2⁻, they were not able to directly present antigens to CD8⁺ T cells. However, we observed a low back-ground of IFN γ production by T cells in presence of tumor cells alone. This bystander T cell activation could be a consequence of the allogeneicity of B cells and their antigen-presenting function that could be further enhanced by sCD40L. By contrast, when tumor cell integrity was affected, this IFN γ production decreased to baseline secretion, as shown in the condition where B cells were necrotic. We also found that whatever the treatment of the tumor cells, macrophages were unable to cross-present viral antigens to anti-influenza T lymphocytes, despite their high engulfment capacity. In these experiments, macrophages appeared to have a strong phagocytic scavenger activity rather than immunostimulating function. By contrast, dendritic cells did effectively cross-present viral antigens from the different lymphoma cell preparations except from the necrotic cells. These results advise against the use of heated tumor cells to pulse DC. So, even if dendritic cells are less efficient at endocytosing than macrophages, this lower uptake seems to be enough to allow the cross-presentation of antigens to CD8⁺ T cells, confirming the great ability of dendritic cells in the cross-presentation of exogenous antigen onto the MHC class-I molecules.

Surprisingly, there was no significant difference in the cross-presentation induced in vitro by dendritic cells loaded with either the non-treated, apoptotic or opsonized cells, even if endocytosis of opsonized cells was more pronounced. However, there are a large number of evidences that Rituximab, the therapeutic monoclonal antibody against CD20, improve the anti tumor immune response, activating the complement system [21, 23-25]. This complement activation could create an inflammatory environment favoring both the recruitment and the activation of dendritic cells and other immune cells. Recently, Franki et al clearly demonstrated in an in vivo B-cell lymphoma model that vaccine consisting in opsonized whole tumor cells cocultured with DC could protected mice from tumor challenge, whereas DC loaded with tumor cells alone were much less effective[26]. So, antibodies against B-cell markers could improve the efficacy of using of whole tumor cells in stimulating the antitumor response in vivo.

Altogether, we suggest here that the use of opsonized whole malignant B cells combined to antigen presenting cells could represent an interesting approach in the setting of clinical trials in the context of non Hodgkin's lymphomas.

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Figure legends

Figure 1: Phagocytosis of tumor cells by antigen-presenting cells. Phagocytosis of lymphoma cells by macrophages (A) or dendritic cells (B) was determined by microscopic counting after a 2-hour incubation. Tumor cells were left untreated (NT), induced in apoptosis by γ -irradiation (APO), in necrosis by heating (NEC), or opsonized by rituximab (OPS). Cells were cyto-centrifuged and stained with May-Grünwald-Giemsa and phagocytosis was evaluated by microscopic counting. The data shown are the mean + SD of 5 experiments, and indicate the percentage of phagocytes containing at least one tumor cell.

Figure 2: Endocytosis of tumor-derived material by antigen-presenting cells. As example, endocytosis by macrophages by flow cytometry is shown at +4°C (A) or +37°C (B). Tumor cells were stained with PKH26 and then added to APC. After two hours, endocytosis was stopped by incubation on ice and by addition of EDTA. The identification of APC was performed with anti-CD11b-FITC and the detection of conjugates between tumor cells and APC was evidenced by anti-CD19 and -CD20 added prior to flow cytometry analysis. Macrophages negative for CD19 and CD20 were gated and analyzed for staining by PKH26. Uptake of tumor-derived material by macrophages (C) and dendritic cells (D) in three independent experiments. The mean percentages (+ SD) of CD11b⁺ PKH26⁺ in CD19⁻CD20⁻ APC are represented for each condition.

Figure 3: Endocytosis of tumor material by macrophages (A) or dendritic cells (B). Tumor cells were stained with PKH26, left untreated, induced in apoptosis, necrosis or opsonized by rituximab and then added to APC labeled with PKH67. After two hours, endocytosis was stopped by incubation on ice and by addition of EDTA and observed under fluorescence microscope.

Figure 4: Treatment of lymphoma B cells with influenza virus. Tumor cells were incubated for 18 hours with inactivated influenza virus. Uptake of viral particles was monitored by detection of (A) viral hemagglutinin by polyclonal rabbit anti-hemagglutinin H1 in B cells treated (bold line) or not (thin line) by virus. (B) Intracellular viral nucleoprotein was detected after permeabilization by acetone and staining with anti-NP, and appears green.

Figure 5: Cross-presentation of viral antigens by dendritic cells to CD8 T cells. Tumor cells were treated with influenza virus and either left untreated (NT), induced in apoptosis (APO), in necrosis (NEC), or opsonized by rituximab (OPS). After extensive washing, B cells were added to dendritic cells for four hours and anti-viral CD8 T cells were then added. Culture supernatant was collected after 48h, and assayed for IFN γ . The data of two experiments are presented.

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