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Olivier Manches, Joël Plumas, Gabrielle Lui, Laurence Chaperot, Jean-Paul Molens, et al.. Anti-Gal-mediated targeting of human B lymphoma cells to antigen-presenting cells: a potential method for immunotherapy using autologous tumor cells.. *Haematologica*, 2005, 90 (5), pp.625-34. inserm-00286462

**HAL Id: inserm-00286462**

**<https://inserm.hal.science/inserm-00286462>**

Submitted on 28 Dec 2010

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# Anti-Gal-mediated targeting of human B lymphoma cells to antigen-presenting cells: a potential method for immunotherapy using autologous tumor cells

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**Background and Objectives.** The residual tumor cells remaining after completion of standard chemotherapy and radiation treatment in B lymphoma patients, may be eradicated by active immunotherapy that stimulates tumor-specific T lymphocytes. Irradiated autologous lymphoma cells expressing tumor-associated antigens (TAA) may serve as a potential tumor vaccine, provided that they are effectively targeted to the antigen-presenting cells (APC). We propose exploiting the natural anti-Gal antibody in order to target vaccinating tumor cells to APC. Anti-Gal constitutes 1% of IgG in human serum and interacts specifically with the  $\alpha$ -gal epitope (Gal $\alpha$ 1-3Gal $\beta$ 1-4GlcNAc-R).

**Design and Methods.**  $\alpha$ -Gal epitopes were synthesized *in vitro* on the membrane of primary lymphoma cells by using the recombinant glycosylation enzyme  $\alpha$ 1,3galactosyl-transferase ( $\alpha$ 1,3GT). Processed tumor cells were opsonized by purified anti-Gal antibodies and studied for uptake (phagocytosis) by APC including monocyte-derived macrophages and dendritic cells. Cross-presentation of tumor antigens after phagocytosis of processed MHC-I negative lymphoma cells was measured by activation of a tumor-specific CD8<sup>+</sup> T-cell line.

**Results.** We demonstrate synthesis of  $\alpha$ -gal epitopes on freshly isolated B lymphoma cells of various types following the use of the recombinant enzyme  $\alpha$ 1,3GT. The subsequent binding of anti-Gal to the *de novo* synthesized  $\alpha$ -gal epitopes opsonizes these tumor cells for effective uptake by macrophages and dendritic cells, through phagocytosis mediated by Fc $\gamma$ R1 (CD64). Moreover, anti-Gal-mediated phagocytosis resulted in cross-presentation of TAA by dendritic cells.

**Interpretations and Conclusions.** This study suggests that immunization with irradiated autologous lymphoma cells processed to express  $\alpha$ -gal epitopes will result in anti-Gal-mediated, *in vivo* targeting of the autologous tumor vaccine to APC.

Key words: lymphoma vaccine, APC targeting, anti-Gal,  $\alpha$ -gal epitope, phagocytosis.

Haematologica 2005; 90:625-634

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**N**on-Hodgkin's B-cell lymphoma represents a heterogeneous group of malignancies in which active immunotherapy may enable the eradication of the residual tumor cells remaining after completion of standard treatment. The demonstration of tumor-specific cytolytic T cells among *in vitro* expanded tumor-infiltrating T lymphocytes<sup>1</sup> suggests that an anti-tumor immune response may be induced in lymphoma patients. In the present study we demonstrate a relatively simple method for preparing autologous tumor vaccines in lymphoma patients that are effectively targeted *in vivo* to antigen presenting cells (APC). This targeting is achieved by exploiting the most abundant natural antibody in humans, the anti-Gal antibody. An effective anti-tumor immune response requires uptake of the vaccinating tumor cells by APC. Tumor

cells in a large undefined proportion of patients are likely to express tumor-associated antigens (TAA). The APC transport the vaccine to the draining lymph nodes, process the tumor antigenic peptides and express these peptides for the activation of tumor-specific T cells. Once activated, these T cells proliferate and differentiate into effector cells that can circulate in the body to detect and destroy tumor cells expressing the vaccinating tumor antigens.<sup>2,3</sup> Vaccination with unmodified autologous lymphoma cells is unlikely to elicit such an immune response because these tumor cells evolve *in vivo* to be *invisible* to APC.<sup>4,5</sup> Targeting of vaccinating tumor cells to APC can be achieved by exploiting Fc $\gamma$  receptors (Fc $\gamma$ R) on all APC, including macrophages and dendritic cells also in their form of Langerhans' cells of the skin. Via these receptors, APC effectively internalize cells

or soluble antigens that are complexed (i.e. opsonized) by an IgG antibody.<sup>6-9</sup> Moreover, binding of antigen-antibody immune complexes to the FcγR of dendritic cells induces effective maturation of these APC, resulting in cross-presentation (cross-priming) of the antigenic peptides by class I MHC molecules for the activation of CD8<sup>+</sup> cytotoxic T cells, and presentation of peptides on class II MHC molecules for the activation of CD4<sup>+</sup> helper T cells.<sup>10-13</sup> Such a 10 to 1000-fold increase in immune response has been observed with immunizing simian immunodeficiency virus (SIV),<sup>14</sup> *Leishmania* vaccine<sup>15</sup> or tumor cells<sup>16</sup> opsonized by the corresponding antibody. A similar targeting strategy for human vaccinating lymphoma cells to APC may be achieved by exploiting the natural anti-Gal antibody.

Anti-Gal is the only natural IgG antibody known to be present in all humans, and constitutes ~1% of circulating IgG.<sup>17</sup> Anti-Gal interacts specifically with α-gal epitopes (Galα1,3Galβ1,4GlcNAc-R) on cell surface glycoconjugates<sup>18,19</sup> and is produced throughout life as a result of antigenic stimulation by bacteria of the gastrointestinal flora.<sup>20</sup> The α-gal epitope is absent in humans, but is abundantly synthesized by the glycosylation enzyme α1,3galactosyltransferase (α1,3GT) on glycolipids and glycoproteins in non-primate mammals, prosimians and in New World monkeys.<sup>21,22</sup> *In vivo*, anti-Gal can bind very effectively to α-gal epitopes, as can be inferred from xenotransplantation studies in which anti-Gal binding to α-gal epitopes on transplanted pig heart or kidney was found to be the main cause for the rapid rejection of such grafts in humans and in Old World monkeys.<sup>19,23</sup> Accordingly, tumor cells engineered to express α-gal epitopes were shown to bind anti-Gal *in vivo* in an experimental animal model and to be destroyed by this antibody.<sup>24</sup> We hypothesized that this binding of anti-Gal to α-gal epitopes on vaccinating autologous tumor cells, processed to express these epitopes, would result in opsonization of such tumor cells and their effective *in situ* targeting to APC.<sup>25,26</sup> In the present study we aimed to determine whether various types of freshly obtained human B lymphoma cells can be enzymatically processed to express α-gal epitopes. We further studied anti-Gal-mediated targeting of such lymphoma cells to human dendritic cells and macrophages and determined whether such targeting results in processing and presentation of TAA peptides on the APC.

## Design and Methods

### Cells

Lymphoma cells were purified from lymph node biopsies of patients with B-cell lymphoma, as described previously.<sup>27</sup> The purity (=97%) of tumor suspensions was evaluated by flow cytometric analysis

and cells were subsequently cryopreserved in liquid nitrogen. Tumor cells were obtained from the following patients: 2 with mantle cell lymphoma (MCL), 6 with follicular lymphoma (FL), and 2 with small lymphocytic lymphoma (SLL), according to the revised European-American classification of lymphoid neoplasms (REAL). Four of the patients were females and 6 were males; their ages ranged from 43 to 76 years old. The mouse myeloma cell line SP2/O, which expresses ~1×10<sup>6</sup> α-gal epitopes per cell,<sup>22</sup> was used as a positive control binding anti-Gal.

Cultures for differentiation of macrophages and dendritic cells were performed using the same process as the MAK<sup>TM</sup> cell processor and VAC<sup>TM</sup> cell processor developed by Immuno-Designed Molecules, SA (Paris) for production of clinical grade macrophages and dendritic cells, respectively, under standard operating procedures.<sup>28</sup> For phagocytosis experiments, macrophages and dendritic cells were generated from healthy volunteers, whereas for cross-presentation, macrophages and dendritic cells were generated from the peripheral blood mononuclear cells (PBMC) of a lymphoma patient whose tumor cells were used to generate an MHC-I deficient B lymphoma cell line (*see the section Assay for cross-presentation*). Macrophages or dendritic cells from healthy or lymphoma patients were phenotypically and functionally very similar.<sup>28</sup> Human macrophages were generated from PBMC by culture in gas-permeable hydrophobic bags (Stedim-Aubagne, France) in 100 mL of Iscove's modified Dulbecco's medium supplemented with 2% autologous plasma, in the presence of 500 U/mL of granulocyte colony-stimulating factor (GM-CSF) (Sandoz, Rueil-Malmaison, France) for 7 days. Immature dendritic cells were generated by cultivating PBMC in RPMI 1640 (Gibco) supplemented with 2% autologous plasma in the presence of 500 U/mL GM-CSF (Sandoz) and 50 ng/mL interleukin (IL)-13 (Sanofi, Labège, France), for 6 days, as previously described.<sup>28</sup> At the end of the culture, cells were purified by centrifugal elutriation, analyzed and cryopreserved. Immunophenotypic analysis confirmed that macrophages were CD14<sup>+</sup>, CD3<sup>-</sup>, CD19<sup>-</sup>, CD56<sup>-</sup>, HLA-DR<sup>+</sup>, CD86<sup>+</sup>, CD1c<sup>-</sup>, CD64<sup>+</sup>, CD32<sup>+</sup>, CD16<sup>+</sup>, CD83<sup>-</sup>, whereas immature dendritic cells grown under these conditions were CD14<sup>-</sup>, CD3<sup>-</sup>, CD19<sup>-</sup>, CD56<sup>-</sup>, HLA-DR<sup>+</sup>, CD86<sup>+</sup>, CD1c<sup>+</sup>, CD64<sup>+</sup>, CD32<sup>+</sup>, CD16<sup>-</sup>, CD83<sup>-</sup>. Prior to phagocytosis experiments, immature dendritic cells were incubated for 48 hours in complete medium supplemented with 10% fetal calf serum (FCS) and 100 ng/mL interferon (IFN)γ (Genzyme, Cambridge, MA, USA) in order to up-regulate CD64.<sup>8</sup>

### Anti-Gal activity of patients' sera

Anti-Gal antibody activity in sera from lymphoma patients and from normal individuals was determined by ELISA. Sera (1:5) were added to a 96-well microtiter plate precoated with 10 µg/mL synthetic  $\alpha$ -gal epitopes linked to BSA (Gal $\alpha$ 1-3Gal,1-4GlcNAc-BSA, designated  $\alpha$ -gal-BSA; Dextra Laboratories, Reading, UK) in carbonate buffer (pH 9.5) and blocked with bovine serum albumin (BSA) 1% in carbonate buffer as previously described.<sup>29</sup> After 2 hours of incubation at room temperature the ELISA plates were washed and anti-Gal binding determined with goat F(ab)'<sub>2</sub> anti-human IgG antibody coupled to horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), followed by color development with orthophenylenediamine (Sigma) and absorbance measured in an ELISA reader at 490 nm (Bio-Tek Instruments Inc., USA). Sera from 11 healthy individuals were included as controls. Specific binding of anti-Gal antibodies was calculated by subtracting non-specific binding measured as absorbance due to IgG binding to BSA coated wells. The specificity of anti-Gal binding to  $\alpha$ -gal-BSA was further confirmed by comparing it with N-acetyllactosamine linked to BSA (Gal $\beta$ 1-4GlcNAc-BSA) (*data not shown*).

### Anti-Gal purification from human serum

Anti-Gal from normal human AB serum was purified by affinity chromatography on a column of synthetic  $\alpha$ -gal epitopes linked to silica beads (Synsorb 115; Chembiomed Ltd., Edmonton, Alberta, Canada) as previously described.<sup>19,30</sup> The specificity of the purified antibody was confirmed in ELISA by its binding to  $\alpha$ -gal-BSA and lack of binding to asialofetuin (Sigma, Saint Quentin Fallavier, France), a fetal bovine glycoprotein lacking  $\alpha$ -gal epitope.

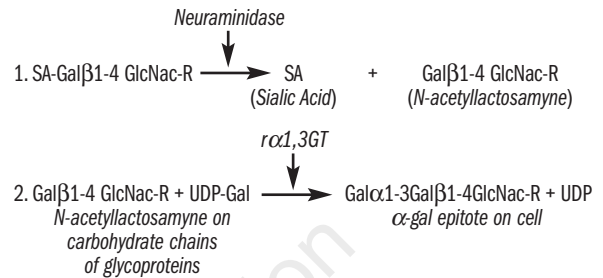
### Recombinant $\alpha$ 1,3galactosyltransferase ( $\alpha$ 1,3GT) production and purification

To produce recombinant (r)  $\alpha$ 1,3GT, the  $\alpha$ 1,3GT gene was cloned from marmoset (New World monkey) cells,<sup>31</sup> tagged with a (His)<sub>6</sub> tail and produced initially in the baculovirus expression system with Sf9 insect cells.<sup>25</sup> In later stages of this study the recombinant enzyme was produced in a yeast expression system of *Pichia pastoris* which secretes the enzyme in the growth medium and thus enables easy purification that yields  $\alpha$ 1,3GT suitable for clinical studies.<sup>32</sup> The enzyme was purified either from the Sf9 cell lysates or from the supernatant of *Pichia pastoris* cultures by affinity chromatography on a nickel-Sepharose column (Probond-Invitrogen) and subsequent elution with 0.2M imidazole.<sup>32</sup>

### Synthesis of $\alpha$ -gal epitopes on lymphoma cells

Complex type N-linked glycoproteins on human cells

contain mostly terminal N-acetyllactosamine residues capped by a sialic acid residue (SA). Therefore, desialylation by neuraminidase allows exposure of acceptor sites (N-acetyllactosamine) for the  $\alpha$ 1,3GT. Subsequently, synthesis of  $\alpha$ -gal epitopes is performed by incubating lymphoma cells with  $\alpha$ 1,3GT, according to the previously described enzymatic reactions.<sup>25,32</sup> The two enzymatic reactions leading to synthesis of  $\alpha$ -gal epitopes are as follows:



Lymphoma cells were thawed and incubated for 30 minutes at 37°C with 5 mU/mL of *Vibrio cholerae* neuraminidase (Boehringer Mannheim, Germany) in Hanks' balanced salt solution (HBSS-Gibco BRL, Cergy Pontoise, France) supplemented with 2% FCS and with 4 mM CaCl<sub>2</sub> added. After washing with saline, cells were incubated for 2 h at 37°C with 30 µg/mL  $\alpha$ 1,3GT in MES buffer pH 6.2 containing 25 mM MnCl<sub>2</sub> and 5 mM UDP-Gal (Boehringer-Mannheim). Modified cells were washed once with saline, then with complete RPMI 1640 medium supplemented by 2% human albumin (LFB, Les Ulis, France). *De novo* synthesized  $\alpha$ -gal epitopes on cell membranes were detected by flow cytometry using the *Griffonia simplicifolia* I-B4 lectin<sup>22</sup> coupled to fluorescein isothiocyanate (FITC) (GS I-B4-Vector Laboratories, Burlingame, CA, USA) in a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA). To analyze the persistence of  $\alpha$ -gal epitope expression on tumor cells, enzymatically processed lymphoma cells were incubated at 37°C in complete RPMI medium supplemented by 10% FCS. At different time points, aliquots were taken and washed in RPMI 1640 supplemented with 2% human albumin. The presence of  $\alpha$ -gal epitopes was monitored by labeling with FITC-coupled lectin on a FACScan flow cytometer. Analysis of human anti-Gal binding to the lymphoma cells was performed by incubation with purified anti-Gal (2 µg/mL) after synthesis of  $\alpha$ -gal epitopes. The secondary antibody-goat anti-human IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) coupled to FITC was then added and fluorescence was analyzed on a FACScan flow cytometer. In order to measure the binding of anti-Gal IgG to lymphoma cells directly, lymphomas were selected for their absence of expression of membrane IgG (e.g. IgM or IgD-

expressing lymphomas). Synthesis of  $\alpha$ -gal epitopes, opsonization and phagocytosis are equally efficient with IgG-expressing lymphoma cells (*data not shown*).

### Anti-Gal-mediated phagocytosis of modified and opsonized tumor cells

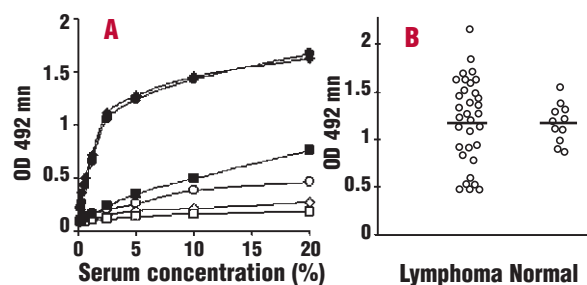
Tumor cells expressing  $\alpha$ -gal epitopes were incubated with purified human anti-Gal or with normal human AB serum (diluted 1:3) for 30 minutes at 4°C. Macrophages or IFN $\gamma$ -treated dendritic cells were then incubated with the opsonized lymphoma cells (APC to tumor cell ratio of 1:3) for 2 hours at 37°C. Aggregates were dissociated by 0.4 mM EDTA for 5 minutes at 37°C. Cells were washed once in RPMI, 2% human albumin and cytocentrifuged preparations were made for visual counting. After May Grünwald Giemsa staining, double-blind counting of phagocytosis was performed on at least 200 macrophages or 400 dendritic cells.

For phagocytosis inhibition studies lymphoma cells were opsonized by purified anti-Gal antibodies for 30 min at 4°C, washed, and incubated with macrophages at 37°C, in the presence of 5  $\mu$ g/mL of anti-Fc $\gamma$ R blocking antibodies (anti-CD64) (mouse IgG1, Diaclone, Besançon, France), anti-CD32 (mouse IgG2b, Stem-Cell Technologies, Grenoble, France), or anti-CD16 (mouse IgG1, Pharmingen). After 2 h, cells were washed and cytocentrifuged then phagocytosis determined as above.

### Assay for cross-presentation

Tumor-specific CD8<sup>+</sup> T cells were generated by stimulation of tumor infiltrating CD8<sup>+</sup> T cells with irradiated autologous lymphoma B cells for three weeks in the presence of IL-1 (1 U/mL), IL-2 (20 U/mL) and IL-12 (1 ng/mL), as we previously described.<sup>1</sup> Cross-presentation of tumor antigens was assessed using a MHC class I-deficient cell line derived from the tumor cells that were grown as a cell line in nude mice (generated by T. Bonnefoy, GRL-EMI 0353, France), established from the lymph node of a patient with follicular lymphoma, as described previously.<sup>33</sup> Nude mice were engrafted with fragments of malignant lymph node, the transplantable tumors were obtained and grown *in vivo* over 4 months. The MHC-I negative cell line (CH1) was established *in vitro* from the tumor cells, and cultured in RPMI medium and 10% de-complemented fetal calf serum. MHC-I negativity was verified by flow cytometry using antibodies against MHC-I or against  $\beta$ 2-microglobulin, and was not modified upon incubation with IFN $\gamma$  (0.1  $\mu$ g/mL).

Synthesis of  $\alpha$ -gal epitopes on the cell line and opsonization by purified anti-Gal antibodies was performed as described above. After extensive washing,  $5 \times 10^3$  B cells were co-incubated with  $1 \times 10^4$  autol-



**Figure 1.** Anti-Gal reactivity in serum from lymphoma patients. **A.** Specificity of the binding to  $\alpha$ -gal-BSA. The sera from three patients (circles, diamonds or squares), were assayed by ELISA for their binding either to BSA (open symbols) or to Gal $\alpha$ 1-3Gal $\beta$ 1-4-BSA ( $\alpha$ -gal-BSA) (closed symbols). Serum concentration is indicated as percentage of the solution. **B.** Sera from normal individuals (n=11) or from patients with FL (n=14), MCL (n=8), DLCL (n=9), SLL (n=5) were tested in ELISA on Gal $\alpha$ 1-3Gal $\beta$ 1-4GlcNAc-BSA ( $\alpha$ -gal-BSA) in microtiter plates. The secondary antibody was peroxidase-coupled anti-human IgG, IgM and IgA, which allowed a color reaction with the peroxidase substrate orthophenylenediamine dihydrochloride and absorbance reading at 492nm. Non-specific binding to BSA was subtracted from binding to  $\alpha$ -gal-BSA. The horizontal bar indicates the mean value for each group.

ogous dendritic cells or macrophages for 4 hours in round-bottom wells, and  $1 \times 10^5$  effector T cells were then added with 100ng/mL soluble CD40 ligand and 1  $\mu$ g/mL enhancer (soluble CD40L kit, Alexis). The culture supernatant was removed after 48h, and assayed for cytokine content using the Cytokine Bead Array kit (Becton Dickinson). As IFN $\gamma$  secretion (between 2 and 8 ng/mL) varied widely between independent experiments, secretion in each condition was calculated as the percentage of maximum secretion which was observed when T cells were co-cultivated with dendritic cells (DC) and opsonized CH1 cells.

Alternatively, as another read-out for cross-presentation, CH1 cells were irradiated (60 Gy) after  $\alpha$ -gal synthesis and before co-culture with APC and CD8<sup>+</sup> T cells. At the end of the 5 day culture,  $37 \times 10^3$  Bq [ $^3$ H]thymidine was added to each well and the cells harvested 18 hours later.

## Results

### Anti-Gal activity in lymphoma patients

A prerequisite for the anti-Gal mediated targeting of lymphoma cell vaccines to APC is the presence of anti-Gal antibodies in the lymphoma patient in titers similar to those in healthy individuals. Binding of anti-Gal antibodies was assayed using Gal $\alpha$ 1-3Gal $\beta$ 1-4-GlcNAc-BSA ( $\alpha$ -gal-BSA) as a solid phase antigen, and compared to binding to BSA alone

**Table 1.** Synthesis of  $\alpha$ -gal epitopes on lymphoma cells (a).

Case	REAL	Without $\alpha$ 1,3-GT (b)		With $\alpha$ 1,3-GT (b)		With $\alpha$ 1,3-GT (b)	
		Without neuraminidase		Without neuraminidase		With neuraminidase	
		%	MFI	%	MFI	%	MFI
1	MCL	2	6	97	651	94	1833
2	MCL	5	5	76	76	98	390
3	FL	6	2	nd	nd	92	364
4	FL	2	3	100	834	99	948
5	FL	2	3	72	342	80	1127
6	FL	5	3	98	248	100	613
7	FL	3	7	nd	nd	91	431
8	FL	4	8	77	393	87	998
9	SLL	2	3	86	76	100	126
10	SLL	1	4	32	42	100	111

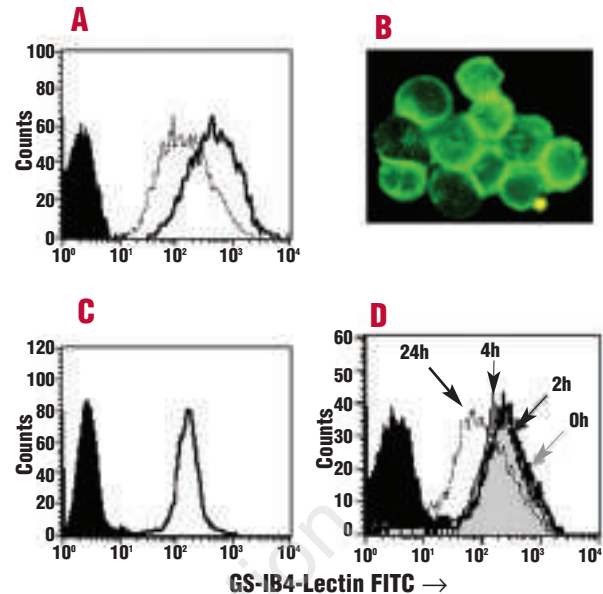
(a) Lymphoma cells were incubated with or without neuraminidase and with or without  $\alpha$ 1,3GT for synthesizing  $\alpha$ -gal epitopes on membrane glycoconjugates. The presence of  $\alpha$ -gal epitopes was analyzed by flow cytometry with FITC-labeled GS-IB4 lectin. Results are shown for ten patients with different types of B lymphoma. (b) Results were expressed as % of positive cells and mean fluorescence intensity (MFI) value.

(Figure 1A). Although heterogeneous, binding of the sera to  $\alpha$ -gal-BSA was specific for  $\alpha$ -gal epitopes, as demonstrated by their much lower non-specific binding to BSA alone. The specificity of the binding was further confirmed by comparing binding of the sera to Gal $\alpha$ 1-3Gal $\beta$ 1-4-GlcNAc-BSA and binding to Gal $\beta$ 1-4GlcNAc-BSA from which the terminal  $\alpha$ -galactosyl is missing (*data not shown*).

Sera diluted 1:5 for 36 patients (14 FL, 8 MCL, 9 DLCL and 5 SLL, according to the REAL classification) were also analyzed for their anti-Gal reactivity in ELISA, with  $\alpha$ -gal BSA (Figure 1B). All patients' sera displayed significant anti-Gal activity. In seven patients the binding yielded an optical density (O.D.) of  $< 0.7$ . However, the mean anti-Gal binding values were similar in the patients and in the healthy group ( $p > 0.05$  – Mann-Whitney non-parametric U test), implying that anti-Gal activity in lymphoma patients does not differ significantly from that in healthy individuals. Therefore, this antibody is likely to bind also *in vivo* to  $\alpha$ -gal epitopes expressed on immunizing lymphoma cells.

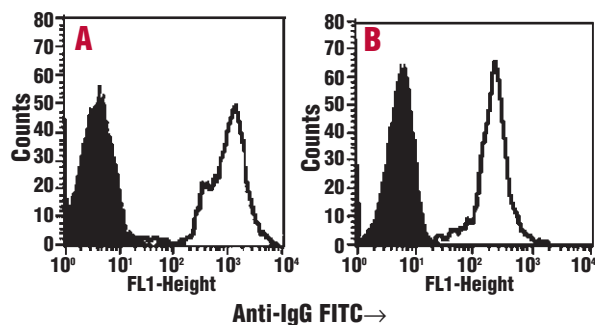
### Synthesis of $\alpha$ -gal epitopes on lymphoma cells

The N-acetylglucosamine residue (Gal $\alpha$ 1-4GlcNAc-R), which is the acceptor for the  $\alpha$ 1 $\beta$ 3GT activity, usually appears on cell surface glycoconjugates either in an uncapped form, or capped with sialic acid (SA-Gal $\beta$ 1-4GlcNAc-R). Sialic acid can be removed by neuraminidase to expose the penultimate N-acetylglucosamine. Thus, it was of interest to determine whether there is a difference in  $\alpha$ -gal epitope synthesis by  $\alpha$ 1 $\beta$ 3GT on untreated lymphoma cells (i.e. on naturally uncapped N-acetylglucosamine residues) or



**Figure 2.** Synthesis of  $\alpha$ -gal epitopes on B lymphoma cells. **A.** Fresh malignant B cells (patient 4 in Table 1) were incubated first with (solid line) or without (dotted line) neuraminidase to remove terminal sialic acid on cell surface glycoconjugates. The cells were then incubated with recombinant  $\alpha$ 1,3galactosyltransferase ( $\alpha$ 1 $\beta$ 3GT), which transfers galactose residues from UDP-Gal to N-acetylglucosamine (Gal $\beta$ 1 $\beta$ 4GlcNAc-R) on the cell membrane. The presence of the epitope was detected by flow cytometry with the GS-IB4 lectin coupled to FITC. Control cells (without enzymatic treatment) are represented by a filled curve. **B.** Modified lymphoma cells were stained with FITC-coupled GS-IB4 lectin and visualized by fluorescence microscopy (original magnification  $\times 60$ ). **C.**  $\alpha$ -gal-positive mouse myeloma cells SP2/O were incubated with GS-IB4 lectin as control. The filled curve represents control unstained cells. **D.** Lymphoma cells were incubated at 37°C after enzymatic synthesis of  $\alpha$ -gal epitopes and assayed at different time points, with GS-IB4 lectin, for the presence of epitopes on the cell surface. Filled curve: no synthesis (identical for all time points). Gray curve: t=0 h, thick line: t=2 h (superimposed), thin line: t=4 h, dotted line: t=24 h after synthesis. One experiment representative of 4 performed on cells from different patients.

on cells treated with neuraminidase (i.e. both on uncapped N-acetylglucosamine residues and on these residues exposed after removal of sialic acid). Like normal human cells, unprocessed lymphoma cells of all types expressed no  $\alpha$ -gal epitopes, as indicated by the lack of GS-IB4 lectin binding. In contrast, 80–100% of lymphoma cells readily bound the lectin after incubation with both neuraminidase and  $\alpha$ 1 $\beta$ 3GT (referred to as  $\alpha$ -gal-lymphoma cells) (Table 1, Figures 2A, B). The binding of GS-IB4 lectin to the enzymatically processed tumor cells was found to be at an intensity which was at least as high as that of binding to murine SP2/O cells (Figure 2C). These malignant mouse B cells express  $\sim 1 \times 10^6$   $\alpha$ -gal epitopes per cell.<sup>29</sup> This implies that incubation of fresh B lymphoma cells with neuraminidase and  $\alpha$ 1 $\beta$ 3GT resulted in the synthesis of  $> 10^6$   $\alpha$ -gal epitope/cell. Tumor cells incubated with both neuraminidase and  $\alpha$ 1 $\beta$ 3GT expressed higher levels of



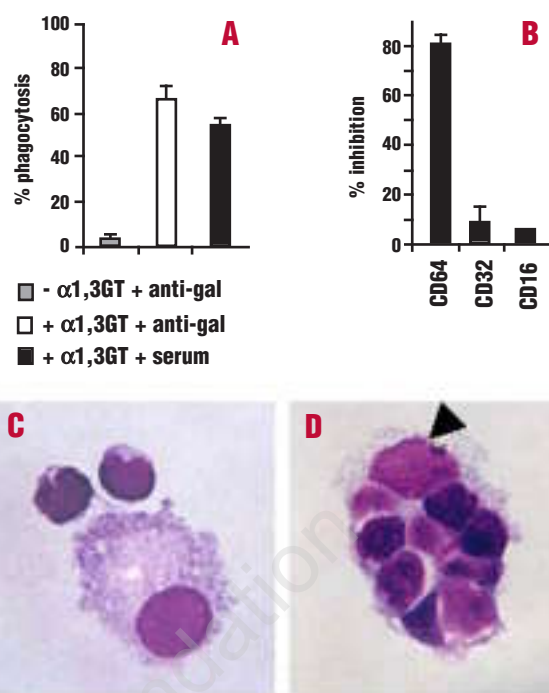
**Figure 3.** Opsonization of modified B cells by anti-Gal antibodies. **A.** After synthesis of  $\alpha$ -gal epitopes, malignant B cells were opsonized by purified anti-Gal antibodies (10  $\mu$ g/mL). The opsonization was revealed by flow cytometry with anti-human IgG antibodies coupled to FITC. Unmodified tumor cells (filled curve) were incubated with purified anti-Gal and anti-IgG-FITC as a negative control. (one representative experiment out of 10). **B.** Positive control of SP2/O cells opsonized by anti-Gal (open curve) prior to addition of FITC-labeled anti-human IgG. SP2/O cells not opsonized by anti-Gal (filled curve).

$\alpha$ -gal epitopes in comparison to cells incubated only with  $\alpha$ 1 $\beta$ 3GT (up to a 5-fold increase, indicated by the mean fluorescence intensity (MFI) values in Table 1 and in Figure 2A). Thus, many of the N-acetyllactosamine residues on lymphoma cells are capped with sialic acid and can be exposed by neuraminidase, thereby increasing the number of synthesized  $\alpha$ -gal epitopes. The synthesis of  $\alpha$ -gal epitopes by  $\alpha$ 1 $\beta$ 3GT was similar when the recombinant enzyme originated from the *Pichia pastoris* expression system or from the expression system of mosquito cells infected by baculovirus containing the  $\alpha$ 1 $\beta$ 3GT gene.

We further determined whether the *de novo* synthesized  $\alpha$ -gal epitopes are expressed on the lymphoma cells for prolonged periods at 37°C.  $\alpha$ -gal epitopes were readily detectable on ~90% of the cells even 24 hours after this synthesis (Figure 2D). The cell viability in this population was found to be >80% (*not shown*). These observations imply a very slow disappearance of  $\alpha$ -gal epitopes due to membrane turnover. The data further suggest that following *in vivo* administration of irradiated lymphoma vaccines, the  $\alpha$ -gal epitopes are likely to remain expressed on the vaccinating lymphoma cells for many hours, thereby allowing their effective interaction with anti-Gal molecules and uptake by APC.

#### Anti-Gal-mediated phagocytosis of $\alpha$ -gal-lymphoma cells by human APC

Binding of human anti-Gal to  $\alpha$ -gal epitopes *de novo* synthesized on lymphoma cells was determined as preliminary evaluation of the tumor cell opsonization that precedes anti-Gal mediated phagocytosis of these cells (Figure 3A). Flow cytometry analysis indi-



**Figure 4.** **A.** Anti-Gal-mediated phagocytosis of lymphoma B cells by human macrophages. Tumor cells were submitted to enzymatic synthesis of  $\alpha$ -gal epitopes on cell membrane (+ $\alpha$ 1,3GT), and subsequently opsonized by purified anti-Gal antibodies (open histogram), or serum (closed histogram). After a 120-min incubation with human macrophages, phagocytosis was scored by visual counting and presented as the percentage of phagocytosing macrophages. Unmodified tumor cells are represented by dashed histograms. Data represent the mean  $\pm$  SD from three different patients. Unmodified tumor cells are not phagocytosed (**C**), whereas modified and opsonized cells are readily phagocytosed by macrophages (**D**). Several tumor cells are phagocytosed by one macrophage. The nucleus of the macrophage is compressed at the periphery of the cytoplasm (arrowhead)(original magnification X100). **B.** Macrophages were incubated with modified and opsonized lymphoma cells, in the presence or absence of Fc $\gamma$ R blocking antibodies (5  $\mu$ g/mL). After 2 hours at 37°C, phagocytosis was scored by visual counting. Inhibition of phagocytosis is presented as the decrease in the proportion of phagocytosing macrophages in the presence of blocking antibodies, as compared to phagocytosis with anti-Gal but no blocking antibodies. Mean inhibition values  $\pm$  SD from two independent experiments are shown.

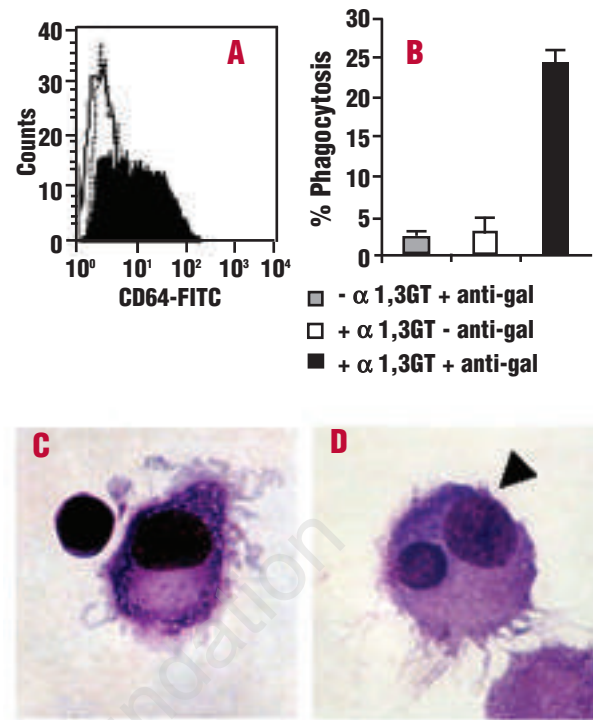
cated that purified anti-Gal antibodies bound to >90% of enzymatically processed lymphoma cells but not to unmodified cells. In accordance with GS-IB4 staining, fluorescence intensity of IgG binding to the modified tumor cells was higher than that to  $\alpha$ -gal epitopes on murine SP2/O cells (Figure 3B). The phagocytosis of lymphoma cells binding anti-Gal was first studied with human macrophages. Lymphoma cells expressing  $\alpha$ -gal epitopes were readily phagocytosed by 70% of the macrophages in the presence of anti-Gal antibodies (Figure 4A), whereas unmodified cells were not phagocytosed, despite the presence of purified anti-Gal antibodies. In the absence of anti-Gal also the processed cells were not phagocytosed (Figure 4C). Thus, phagocy-

tosis of the processed lymphoma cells occurred only after opsonization by anti-Gal antibodies. The macrophages were found to internalize 1-8 tumor cells (Figure 4D). A similar phagocytosis was observed when the processed lymphoma cells were incubated with heat inactivated serum instead of anti-Gal (Figure 4A). This implies that the anti-Gal within the serum is capable of opsonization of the processed tumor cells as well as the purified anti-Gal. To study the receptors involved in anti-Gal mediated phagocytosis by macrophages, we studied phagocytosis of processed lymphoma cells in the presence of antibodies that block the 3 types of Fc $\gamma$ R. Incubation of macrophages with anti-CD64 (high affinity receptor Fc $\gamma$ RI) antibodies inhibited most of the observed phagocytosis, whereas anti-CD32 (Fc $\gamma$ RII) and anti-CD16 (Fc $\gamma$ RIII) had no significant effect on the phagocytosis (Figure 4B). These data suggest that Fc $\gamma$ RI has a predominant role in triggering the phagocytosis of cells opsonized by anti-Gal antibodies.

Since dendritic cells are very potent APC, it was of interest to determine their capacity to phagocytose anti-Gal-opsonized  $\alpha$ -gal-lymphoma cells. In contrast to freshly isolated blood dendritic cells,<sup>8</sup> *in vitro* monocyte-derived dendritic cells (mo-DC) do not express the Fc $\gamma$ RI receptor for IgG.<sup>28</sup> In view of the significance of Fc $\gamma$ RI in macrophage phagocytosis of anti-Gal-opsonized cells, and since the mo-DC did not phagocytose anti-Gal opsonized lymphoma cells (*data not shown*), we up-regulated expression of Fc $\gamma$ RI on mo-DC by culturing them in the presence of IFN $\gamma$  for 48 hours. This incubation significantly increased the expression of CD64 on dendritic cells (50% of cells became positive) (Figure 5A), similar to a previous report<sup>8</sup> but had no effect on the expression of CD1a, CD14, CD16, and CD32 (*not shown*). The IFN $\gamma$ -treated mo-DC could phagocytose anti-Gal opsonized lymphoma cells (20% of phagocytosing dendritic cells, in most cases internalizing one tumor cell) (Figures 5B and 5D). Unmodified lymphoma cells incubated with anti-Gal were not phagocytosed (Figure 5B and 5C), because this antibody cannot opsonize the cells in the absence of  $\alpha$ -gal epitopes. Similarly, processed lymphoma cells expressing  $\alpha$ -gal epitopes which were incubated with IFN $\gamma$ -treated mo-DC in the absence of anti-Gal were not phagocytosed (Figure 5B), implying that the phagocytosis is mediated by anti-Gal binding to these epitopes.

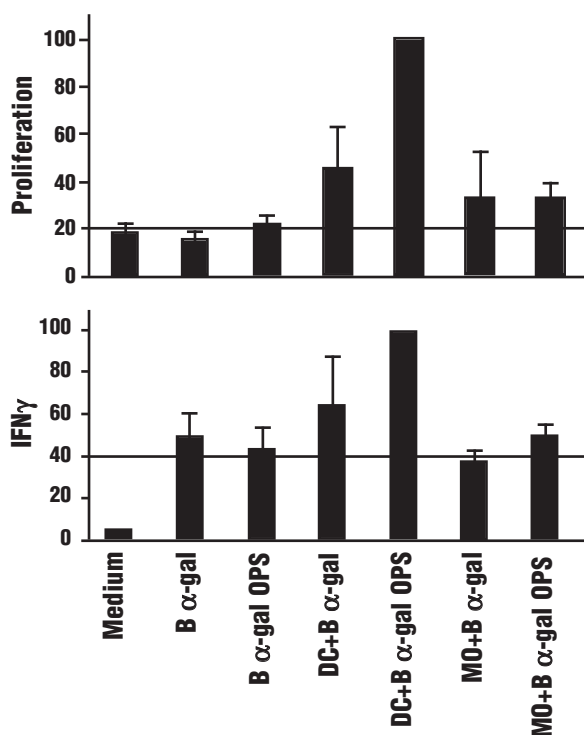
#### Anti-Gal-mediated cross-presentation of tumor antigens by dendritic cells

The observed anti-Gal-mediated phagocytosis of  $\alpha$ -gal lymphoma cells processed to express  $\alpha$ -gal epitopes raised the question of whether this phagocytosis affects cross-presentation of TAA by dendritic cells and macrophages. Since lymphoma B cells stim-



**Figure 5.** Anti-Gal-mediated phagocytosis of lymphoma B cells by immature dendritic cells. **A.** Incubation of immature dendritic cells with IFN $\gamma$  up-regulates expression of CD64. Immature dendritic cells generated from blood monocytes in GM-CSF and IL-13 were incubated for 48 hours with 100ng/mL IFN $\gamma$ . The expression of CD64 was monitored by flow cytometry on dendritic cells at day 0 (dotted line) or after 48 hours with IFN $\gamma$  (filled curve). Thin line: isotype control on dendritic cells incubated 48 h with IFN $\gamma$ . **B** to **D**) Phagocytosis of anti-Gal-opsonized lymphoma cells. Tumor cells were submitted to enzymatic synthesis of  $\alpha$ -gal epitopes on cell membrane (+ $\alpha$ 1,3GT), and subsequently opsonized (+anti-gal) or not (-anti-gal) by purified anti-Gal antibodies. After 2 hours of incubation with dendritic cells, phagocytosis was scored by visual counting and represented as the percentage of phagocytosing dendritic cells. Data represent the mean  $\pm$  SD from two different patients. Modified lymphoma cells are phagocytosed by dendritic cells in the presence of anti-Gal (**D**) whereas unprocessed cells are not (**C**). The dendritic cell nucleus is indicated by an arrowhead (original magnification X100). One representative experiment out of four independent ones on cells from different patients.

ulate APC when co-cultured with T lymphocytes,<sup>27</sup> cross-presentation of tumor antigens by autologous dendritic cells or macrophages was assessed by using a MHC class I-deficient cell line (CH1) derived from the primary tumor cells. In this setting, tumor cells cannot present tumor antigens to CD8<sup>+</sup> T lymphocytes, and CD8<sup>+</sup> T-cell activation reflects cross-presentation of tumor antigens by HLA-I-expressing APC. We measured the proliferation and IFN $\gamma$  secretion of the CD8<sup>+</sup> T lymphocytes upon co-culture of lymphoma cells expressing  $\alpha$ -gal epitope and opsonized by anti-Gal, with autologous macrophages or dendritic cells. As shown in Figure 6, cross-activation of CD8<sup>+</sup> T-cells by mo-DC co-incubated with  $\alpha$ -gal-expressing B lymphoma cells (DC+B  $\alpha$ -gal) increased significantly when these tumor cells were opsonized by anti-Gal (DC+B  $\alpha$ -gal OPS). This was



**Figure 6.** Anti-Gal-mediated cross-presentation of tumor antigens by dendritic cells. Macrophages (MO) or dendritic cells (DC) were incubated with opsonized MHC-class I deficient lymphoma B cells expressing  $\alpha$ -gal epitopes. Cross-presentation was measured as proliferation ( $^3\text{H}$ -thymidine incorporation, upper panel) or IFN $\gamma$  secretion (lower panel) by anti-tumor CD8 $^+$  T lymphocytes upon incubation in medium, or with opsonized (B  $\alpha$ -gal OPS) or unopsonized (B  $\alpha$ -gal)  $\alpha$ -gal lymphoma cells, or with MO or DC phagocytosing  $\alpha$ -gal lymphoma cells, opsonized (MO/ DC+ $\alpha$ -gal OPS) or not (MO/ DC+ $\alpha$ -gal). Results are presented as mean percentage ( $\pm$ SD) of the response measured in the condition DC+B  $\alpha$ -gal OPS in two independent experiments.

indicated by the ~5-fold increase in T-cell proliferation in comparison to proliferation measured in the absence of anti-Gal. Moreover, cytokine secretion indicated a ~2.5-fold increase in secretion in the cell cultures of DC+B  $\alpha$ -gal OPS (i.e. cultures containing tumor cells were opsonized by anti-Gal) in comparison to non-opsonized tumor cells expressing  $\alpha$ -gal epitopes (DC-B  $\alpha$ -gal). No parallel increase was observed with macrophages. Only low cross-activation occurred in the presence of unopsonized  $\alpha$ -gal-CH1 alone, whereas significant activation required opsonization of lymphoma cells by anti-Gal antibodies, suggesting that cross-presentation involved Fc $\gamma$ R.

## Discussion

The deficient uptake, processing and presentation of TAA by APC has been proposed as a major obstacle for immune activation against tumors.<sup>4</sup> In the case of B-cell lymphoma this deficiency is more pro-

nounced since the tumor cells proliferate within the histological site of the immune system, i.e. within the lymph nodes. This implies that most lymphoma cells are *regarded* by APC as normal cells and therefore evade internalization and processing by the APC. The present study describes a simple and clinically feasible method for inducing effective uptake of vaccinating autologous lymphoma cells by APC. This is achieved by enzymatic processing of the lymphoma cells to express  $\alpha$ -gal epitopes and the subsequent *in situ* opsonization of the vaccinating tumor cells by the natural anti-Gal antibody. The main receptor on APC that enables the cells to identify any particulate or soluble material as an antigen that must be internalized and processed is the Fc $\gamma$ R, binding IgG molecules that opsonize the antigen. Targeting of antigens, such as ovalbumin or tetanus toxoid complexed with the corresponding antibodies to Fc $\gamma$ R of APC, was reported to increase CD4 $^+$ , CD8 $^+$  cells and antibody response by up to 1000 fold, in comparison to the effect of similar amounts of antigen, in the absence of an antibody.<sup>13,34,35</sup> Similarly, targeting of surrogate tumor antigens to APC by antibody complexing can confer immune resistance to the live tumor cells at a level which is much higher than that of uncomplexed tumor antigens.<sup>34</sup> The anti-Gal IgG antibody, which is the only known antibody that is produced in large amounts in all humans can serve for targeting any B lymphoma vaccine, as well as other vaccinating tumor cells to APC, provided that the vaccinating cells are processed to express  $\alpha$ -gal epitopes. In the present study we demonstrated that the level of anti-Gal activity in B lymphoma patients is similar to that found in healthy individuals. We further demonstrated the effective synthesis of  $> 10^6$   $\alpha$ -gal epitopes per lymphoma cell by the use of neuraminidase and  $\alpha$ 1 $\beta$ 3GT. The opsonization of the processed lymphoma cells by anti-Gal results in effective Fc $\gamma$ RI mediated phagocytosis of these tumor cells by macrophages and dendritic cells. We could further demonstrate high expression of  $\alpha$ -gal epitopes on the processed lymphoma cells even after 24 hours of incubation of these cells at 37°C. The minimal decrease in  $\alpha$ -gal epitope expression is likely to be the result of turn over of the cell membrane. Since the immunizing lymphoma cells will be lethally irradiated prior to vaccination, in order to prevent *in vivo* proliferation of the tumor cells, the metabolic activity in the irradiated cells will be residual. Therefore, the membrane turn-over will be minimal and *in vivo* loss of  $\alpha$ -gal epitopes is likely to be residual. Thus, the vaccinating lymphoma cells expressing this epitope are likely to retain the  $\alpha$ -gal epitope *in vivo* for periods long enough to bind anti-Gal and to be internalized by APC. In addition to effective targeting of antigens to APC, the internalization of opsonized

antigens via the FcγR was reported to stimulate the differentiation of immature dendritic cells into mature dendritic cells that cross-present the processed antigen on MHC class I molecules, thus activating both CD8<sup>+</sup> T cells (by antigenic peptides presented on MHC class I molecules) and CD4<sup>+</sup> T cells (by antigenic peptides presented on MHC class II molecules).<sup>10,12-15,36</sup> These two types of presentation can be facilitated also by anti-Gal opsonized antigens. In a previous study we demonstrated that anti-Gal opsonization of influenza virus expressing α-gal epitopes increases activation of virus-specific CD4<sup>+</sup> T cells via MHC class II presentation by >10 fold.<sup>37</sup> This potentiation of CD4<sup>+</sup> T-cell activation by anti-Gal may further facilitate cross-priming of functional CD8<sup>+</sup> T cells, as activated CD4<sup>+</sup> T cells are crucial for providing *help* to cross-primed CD8<sup>+</sup> T cells, through CD40L-dependent and -independent pathways.<sup>34,38</sup> Potentiation of CD4<sup>+</sup> T-cell activation by anti-Gal antibodies may therefore be crucial for the functionality of cross-primed CD8<sup>+</sup> T cells. In the present study we demonstrate increased activation of tumor-specific CD8<sup>+</sup> T cells by dendritic cells internalizing anti-Gal-opsonized lymphoma cells. Although we demonstrated cross-presentation of lymphoma cell antigens on dendritic cells, but not on macrophages from the same individual, under optimal *in vivo* conditions, cross-presentation may also be achieved with macrophages that internalized the anti-Gal-opsonized vaccinating tumor cells.

The efficacy of anti-Gal opsonized tumor vaccines in eliciting a protective anti-tumor immune response *in vivo* has been demonstrated in studies in α1β3GT knock out mice (i.e. mice lacking α-gal epitopes and producing anti-Gal), immunized with irradiated melanoma cells expressing α-gal epitopes and challenged with these melanoma cells which, however, lacked α-gal epitopes.<sup>29</sup> Challenge with live tumor resulted in 100% tumor growth in the control mice (i.e. mice immunized and challenged with melanoma cells lacking α-gal epitopes). In contrast, more than a third of the mice immunized with tumor cells expressing α-gal epitopes developed a protective immune response that prevented the growth of the tumor administered in the challenge. Moreover, mice immunized with tumor cells expressing α-gal epitopes and which developed tumors, displayed an inflammatory response comprised of CD4<sup>+</sup> and CD8<sup>+</sup> T cells and macrophages that surrounded the tumor. However, in control mice immunized with tumor cells lacking α-gal epitopes the immune system ignored the growing tumor. Although the precise mechanisms of the protective immune response elicited by immunization with anti-Gal-opsonized tumor cells is not clear as yet, it is probable that such immunization involves elevated activation of both CD4<sup>+</sup> and CD8<sup>+</sup> T cells. The recent demonstration

of increased priming of CD8<sup>+</sup> T cells responses against visceral leishmaniasis by natural antibodies binding to the protozoal vaccine<sup>15</sup> and the increased activation of both specific CD4<sup>+</sup> and CD8<sup>+</sup> T cells in mice receiving dendritic cells pulsed with ovalbumin/anti-ovalbumin immune complexes,<sup>34</sup> both suggest that a similar scenario may occur in anti-Gal producing mice that are immunized with tumor cells processed to express α-gal epitopes. The processing of B lymphoma cells to express >1×10<sup>6</sup> α-gal epitopes/cell is achieved by a 2h enzymatic reaction. As many as 1-3×10<sup>9</sup> tumor cells can be processed in this reaction in an enzyme reaction volume of no more than 30 mL. In view of the effective anti-Gal-mediated targeting of the processed lymphoma cells to APC and the various experimental animal models indicating that such opsonized vaccines are likely to have increased immunogenicity *in vivo*, we propose to use such lymphoma cells for immunizing patients against their autologous tumor antigens. Since every patient receives his/her own processed and irradiated tumor cells as a vaccine, the tumor antigens on the vaccinating cells are *customized* for each patient and there is no need to characterize these antigens. Such a vaccine may be given to patients achieving remission following treatment with standard protocols. Moreover, this vaccine may be administered to lymphoma patients who recover from stem cell transplantation in order to eradicate any tumor cells that may remain as minimal residual disease.

Thus, immunization with autologous tumor cells processed to express α-gal epitopes may be regarded as an adjuvant immunotherapy given in addition to any of the currently used treatment protocols. In some of the vaccinated patients, the immune response against tumor antigens may be strong enough to result in the eradication of tumor cells remaining in a significant proportion of lymphoma patients and causing relapse of the disease after completion of standard treatment protocols. Phase I clinical trials for studying the safety of this immunotherapy treatment modality have been approved by the FDA for use in patients with recurrent malignancies (IND-183111, U. Galili sponsor). If this vaccine is found to be safe, future phase II studies will be performed to determine the efficacy of the autologous tumor vaccine in patients in first remission.

OM, JP and UG: designed the study and wrote the paper; OM, JP, GL, JPM: carried out the analyses; LC, JCB: analyzed data; JJS: provided with tumor cells and brought clinical information. JP taken primary responsibility for the paper. The authors declare that they have no potential conflict of interest.

This study was supported by a NATO grant CRG972123 to J.P. and by NIH grant CA85868 to U.G.; grants n°5555 from "Association pour la Recherche contre le Cancer", from "Ligue contre le Cancer" comités Isère et Savoie.

Manuscript received January 21, 2005. Accepted April 6, 2005.

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