ERJ Express. Published on May 3, 2011 as doi: 10.1183/09031936.00160210

Recognition of pleural mesothelioma by MUC1(950-958)/HLA-A*0201 specific CD8+ T lymphocyte

David Roulois[1,2], Virginie Vignard[1], Fabien Gueugnon[1], Nathalie Labarrière[1,2], Marc Grégoire[1,2] and Jean-François Fonteneau[1,2]

[1] INSERM, U892, Institut de Recherche Thérapeutique, France
[2] Université de Nantes, Faculté de médecine, France

Corresponding author: Jean-François Fonteneau, PhD
INSERM, U892, Institut de Recherche Thérapeutique, Université de Nantes,
8 quai Moncousu, BP70721, 44007 Nantes Cedex1, France
Phone: (+33) 2.28.08.02.39
Fax: (+33) 2.28.08.02.04
Email: jean-francois.fonteneau@inserm.fr

Running Title : MUC1 recognition on mesothelioma by CD8+ T cell

Abstract

Recent clinical investigations have demonstrated that T cell based immunotherapy of Malignant Pleural Mesothelioma (MPM) could represent an alternative to the other therapeutic strategies. However, its development suffers from the lack of identified tumor antigenic targets. Mucin1 (MUC1), which is expressed and recognized by cytotoxic T cells in numerous cancer types, is not investigated as a potential immune target in MPM. Thus, the objective of this study was to analyze MUC1 expression by MPM and to determine if this antigen can be the target of cytotoxic CD8+ T cells (CTL).

We first evaluated the expression and glycosylation of MUC1 by MPM cell lines using different MUC1 specific monoclonal antibodies. We then obtained a CTL clone specific of MUC1(950-958) peptide/HLA-A*0201 and studied its IFN-γ and cytotoxic response against MPM cell lines.

We found that all MPM cell lines expressed MUC1 protein at the cell surface with different glycosylation profiles. We also evidenced that HLA-A*0201+ MPM cell lines are recognized and lysed by a HLA-A*0201/MUC1(950-958) specific CTL clone independently of MUC1 glycosylation profile.

Thus, MUC1 expression and antigen presentation by MPM cells may represent an attractive target for immunotherapeutic treatment of MPM despite its hyperglycosylated profile.

Keywords: cytotoxic T cells, glycobiology, mesothelioma, mucin1, immune response

Introduction

Malignant pleural mesothelioma (MPM) is an aggressive tumor of the pleura, usually associated with chronic asbestos exposure. Incidence is increasing and is expected to peak around the year 2020 in western world, and to continue to rise in developing countries [1].

Clinical strategies actually developed in MPM treatments including chemotherapy, radiotherapy and surgery, are of limited efficacy [2]. However, MPM case reports and recent clinical trials describe the use of T cell based immunotherapy as an interesting alternative in mesothelioma treatment [3]. Indeed, previous observations have demonstrated evident correlation between presence of lymphocytes infiltrate with better prognosis [4-7]. Furthermore, we previously showed, in preclinical studies, that CTL can be generated against MPM [8, 9]. More recently, Hegmans et al reported that injection to patients of dendritic cells (DC) pulsed with autologous tumor cell lysate is capable of inducing cytotoxic T cell response against MPM [10].

The limit to the development of T cell based immunotherapeutic treatments of MPM is the lack of well characterized tumor-associated antigens (TAA) recognized by T cells. In literature, recognition of MPM cells by TAA specific CTL has been rarely described and not analyzed in details. Yokokawa et al showed that a CTL line specific for mesothelin(547-556)/HLA-A*0201 was able to lyse three mesothelin+ HLA-A*0201+ MPM cell lines [11], and similarly, an investigation developed by May et al showed that a CTL line specific to Wilms' tumor 1 Oncoprotein(122-140)/HLA-A*0201 was able to lyse one WT1+ HLA-A*0201+ MPM cell line [12].

An additional TAA now evidenced of interest is the Mucin-1 (MUC1) antigen. This highly glycosylated type I transmembrane glycoprotein, with a variable number of 20 amino acid repeat sequences referred to as "variable number tandem repeat" (VNTR), is now described as one of the most interesting target for cancer immunotherapy [13, 14]. It was originally reported that VNTR sequence can be specifically recognized by cytotoxic CD8+ T lymphocytes on the surface of numerous cancer cell types (breast, pancreatic cancer, and multiple myeloma) in a HLA class I unrestricted-fashion [15]. This recognition was dependent of a tumor specific hypoglycosylation profile of MUC1 [16, 17], that is not present in normal cells [18]. More recently, classical recognition of MUC1 peptides in association with HLA class I molecules on the surface of tumor cells by CTL has also been demonstrated. One HLA-A1 restricted and several HLA-A*0201 restricted epitopes of MUC1, notably MUC1(950-958), recognized by CTL on the surface of tumor cells have been described [19-22], but not investigated in MPM.

Up to date, it is known that MUC1 is overexpressed by MPM compared to normal mesothelioma cells [23]. Thus, MUC1 could represent an attractive TAA to target CTL responses against MPM. To assess this presentation, we analyzed MUC1 expression and glycosylation by MPM cells and we obtained different MUC1(950-958)/HLA-A*0201 specific CTL clones from PBMC of a HLA-A*0201+ healthy donor and study their response against MPM cell lines. We found that the most highly reactive CTL clone recognized and lysed HLA-A*0201+ MPM tumor cells, independently of MUC1 glycosylation profile. This result suggests that MUC1 may be a good TAA candidate for the development of T cell based immunotherapy of MPM.

Materials and methods

Tumor cell culture

Pleural effusions were collected by thoracocentesis and diagnosis was established by immunohistochemical and immunocytochemical labelling. All patients gave signed and informed consent. Human MPM cell lines (Meso4, Meso13, Meso34, Meso35, Meso45, Meso47, Meso56, Meso62, Meso96, Meso122, Meso144, and Meso148) were obtained from pleural effusions. They were established and characterized for several specific markers in our laboratory. The method of isolation of these cell lines is described elsewhere (Gueugnon F. *et al*, "Identification of novel markers for the diagnosis of malignant pleural mesothelioma", in press in *American Journal of Pathology*). They all displayed an epithelioid phenotype. Human breast cancer cell lines MDA-MB231 (established by R. Cailleau) and MCF-7 (established by B.J. Sugarman) were obtained respectively from D. Jäger (Klinik für Oncologie, Zürich, Switzerland) and from ATCC (Manassas, VA, USA). Cell lines were maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS, 100U/mL penicillin, 0.1mg/mL streptomycin and 2mM L-Glutamine (Sigma-Aldrich, France). The T2 cell line (Gift of T. Boon, Ludwig Institute for Cancer Research, Brussels) is a HLA-A*0201+ human T cell leukemia/B cell line hybrid defective for TAP1 and TAP2, thus expressing empty HLA class I molecules at its surface that can be loaded with exogenous peptide [24]. Cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C. In some experiments, tumor cell lines were cultured for 24h with 500IU/ml IFN-γ (Abcys, France) or 48h with 5mM of BGN (Benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside, Sigma Aldrich) during the first 24h.

Antibodies and peptides

PE-conjugated mouse anti-human IFN-γ mAb, mouse anti-human HLA-A2 mAb (clone BB7.2) and FITC-conjugated mouse IgG1k Isotype control mAb were purchased from BD (Le Pont-De-Claix, France). Mouse anti-human MUC-1 (clone HMFG-1 and SM3) mAbs were purchased from Abcam (Paris, France). Mouse anti-human MUC-1 (clone VU-3C6) mAb was purchased from Santa Cruz Biotechnology (Tebu-bio, France). FITC-conjugated anti-human CD58 mAb, FITC-conjugated anti-human HLA-ABC mAb and PE-conjugated Goat $F(ab')^2$ Fragment Anti-Mouse IgG (H+L) were purchased from Beckman Coulter (Roissy CDG, France). FITC-conjugated anti-Human CD54 mAb was purchased from R&D Systems (Lille, France). MUC1(950-958), STAPPVHNV, and Mesothelin(530-538), VLPLTVAEV, peptides were purchased from Eurogentec (Angers, France). Peptides were at least 95% pure.

T cell priming

Blood from HLA-A*0201+ healthy donors was obtained from the "Nantes, Etablissement Français du Sang". Induction of MUC1(950-958)/HLA-A*0201+ specific CD8+ T cells was performed as we described previously with minor modifications [25]. Briefly, T lymphocytes were co-cultured in RPMI1640 supplemented with 8% pooled human serum (pHS) produced locally with monocytes derived dendritic cells (DC) differentiated 5 days with 1000IU/ml GM-CSF (Abcys) and 200U/ml IL-4 (Abcys), then matured 24hrs with 50μg/ml PolyI/C (Sigma) and 20ng/ml TNF-α (Abcys), and pulsed 2h with 10 μM of MUC1(950-958) peptide. T cell cultures were restimulated weekly with peptide-pulsed DCs in presence of 10U/ml IL-2 (Proleukin, Chiron Corp.) and 5ng/ml IL-7 (R&D systems). Six days after the third stimulation, an aliquot of each T cell culture was used to evaluate the percentage of MUC1(950-958) specific T cells by IFN-γ intracytoplasmic staining.

T cell clones

Cells from polyclonal cultures containing MUC1(950-958) specific T cells were cloned by limiting dilution as we previously described [25]. Briefly, T cells were plated in U-bottom 96 well plates with irradiated (35gray) feeder cells ($1x10^5$ allogenic PBMCs and $1x10^4$ Epstein Barr virus-transformed B lymphocyte cells/well), at concentrations of 10, 1, or 0.5 T cells/well. The stimulatory medium consisted of RPMI1640 containing 8% pHS, 150U/ml IL-2 and 1µg/ml Phytohemagglutinin-L (PHA-L, Sigma). After 2 weeks, each clone was tested for peptide specificity. Specific clones were maintained in culture by periodic restimulation.

CDR3b sequencing

RNA from $5x10^6$ cells from each T cell clone was extracted with RNable reagent (Eurobio, Ulis, France) according to the supplier's instructions and dissolved in 15µl of water. Reverse transcription, PCR amplification and sequencing were performed as described [26]. We have followed the TCR nomenclature established by Arden et al [27].

Immunofluorescence and flow cytometry

For membrane staining, $1x10^5$ cells were incubated at 4°C for 30min with 1µg/ml of specific or isotype control mAb and washed. mAb dilution and washing were performed using PBS containing 0.1% BSA (Sigma). When non-conjugated mAb were used, a second incubation with PE-conjugated Goat $F(ab')^2$ Fragment Anti-Mouse IgG (H+L) was performed. Fluorescence was analyzed by flow cytometry (FacsCalibur, BD Biosciences), using Cellquest software (BD Biosciences). Relative fluorescence intensity (RFI) was calculated as the sample mean fluorescence divided by isotype control mean fluorescence.

For IFN-γ intracytoplasmic staining, T2 or tumor cell lines were plated at $1x10^5$cells/well in a 96-well plate. Beforehand, cells were pulsed with different concentrations of MUC-1(950-958) for 1h at 4°C and then washed. They were co-cultured with $5x10^4$ of MUC1 specific CD8+ T cell clone in media containing 10µg/mL brefeldin A (Sigma) for 6h at 37°C. Cells were then fixed with PBS containing 4% paraformaldehyde for 10min at RT. Cell membranes were permeabilized with PBS containing 0.1% BSA and 0.1% saponin and incubated with PE-conjugated mouse anti-human IFN-γ mAb for 30min at room temperature and then washed. Production of IFN-γ was determined by flow cytometry with a gate on T cells (FacsCalibur, BD Biosciences).

Intracellular Ca2+ level video imaging

Measurement of the intracellular Ca2+ levels were performed within CD8+ T cell clone N5.14 loaded with 1mM Fura-2/AM(Molecular Probes, Invitrogen, France) for 1h at room temperature in HBSS. T cells were washed, resuspended in HBSS with 1%FCS and seeded on Lab-Tek glass chamber slides (Nunc, Naperville, IL) coated with poly-L-lysin (Sigma-Aldrich). T cells were cocultured with tumor cells that were left to adhere on glass slides for 1h at 37°C before addition of T cells. Measurements of intracellular Ca2+ responses were performed at 37°C with a DMI 6000 Bmicroscope (LeicaMicrosystems,Nanterre, France). Cells were illuminated every 15sec with a 300W xenon lamp by using 340/10nm and 380/10nm excitation filters. Emission at 510nm was used for analysis of Ca2+ responses and captured with a Cool SnapHQ2 camera (Roper, Tucson,AZ) and analyzed with Metafluor 7.1 imaging software (Universal Imaging, Downington, PA).

$Na_2{}^{51}CrO_4$ Cytotoxic assay

Tumor cell lines were incubated with $Na_2{}^{51}CrO_4$ (PerkinElmer, Boston, USA) for 1h at 37°C. $1x10^3$ tumor cells (target) were then washed and co-cultured with MUC1 specific

CD8+ T cell clone (effector) in a 96-well plate for 4h at 37°C in triplicate. Effector:target (E:T) ratios 2:1; 10:1 and 50:1 were used. After a 4h- incubation at 37°C, 25µl of each supernatant was collected and added to 100µl scintillation liquid cocktail (OptiPhase "Supermix", PerkinElmer, Boston, USA) before liquid scintillation counting. The percentage of specific lysis was calculated as follows: 100 x (experimental release – spontaneous release)/(maximum release – spontaneous release). The spontaneous release of ^{51}Cr was determined from target cells cultured alone. The maximum release of ^{51}Cr was obtained from target cells which were lysed in media containing 1%Triton X-100 (Sigma).

Results

MUC1 expression by MPM cells

We first performed a real-time PCR experiment to determine if the *MUC1* gene was transcribed. Variable levels of MUC-1 transcript were detected in all MPM lines tested (data not shown). We then studied using flow cytometry the expression of MUC1 protein and some molecules involved in peptide presentation and T cell activation (CD54, CD58 and HLA class I) on the surface of a large collection of MPM cells lines (fig.1). We used a combination of three monoclonal antibodies that distinguishes different glycosylation state of MUC1: HMFG-1, SM3 and VU-3-C6 [28]. Clone HMFG-1 recognizes glycosylated and hypoglycosylated forms of MUC1, whereas clones SM3 and VU-3-C6 are specific of hypoglycosylated forms. Furthermore, these antibodies recognized the MUC1 VNTR motif which is a 20 amino acids repeated sequence whose number varies from 20 to 125 repeats depending of the MUC1 alleles expressed. Thus staining intensity with these antibodies not only reflects the quantity of MUC1 at the cell surface, but is also dependent of the number of VNTR present in MUC1. Using the HMFG-1 mAb, we observed that MUC1 is expressed on the surface of all MPM cell lines tested (fig.1). However, the staining level is variable among MPM cell lines with relative fluorescence intensity (RFI) ranging from 7.6 for Meso47 to 105.2 for Meso56. These staining levels are slightly lower to the one observed for MCF-7, a breast cancer cell line known to be recognized by Mucin-1 specific CD8+ T cells [20]. More staining heterogeneity was observed using the two mAb specific for hypoglycosylated forms of MUC1. Some MPM cell lines, such as Meso35, Meso47, Meso96 and Meso148 were negative or slightly stained (RFI<2), whereas other MPM cell lines, such as Meso13 and Meso56 were more markedly stained (RFI>5). Furthermore, some MPM cell lines were stained preferentially by one of the two mAb specific of different hypoglycosylated forms, such as Meso56 and Meso122 which are preferentially stained by SM3 and VU-3-C6 respectively. Altogether, these results suggest that all MPM cell lines express MUC1 with differences in the level and the type of glycosylation.

We also analyzed the expression of molecules implicated in CD8+ T cell activation, such as HLA-ABC (HLA class I), CD54 (ICAM-1) and CD58 (LFA-3) on MPM cells. All MPM cell lines were stained positively for these molecules with the exception of Meso34 which expressed a low level of CD54 molecules (fig.1). Thus all MPM cell lines seem to be equipped to activate a MUC1 specific CD8+ T cells response.

*HLA-A*0201 restricted MUC1(950-958) specific CD8+ T cell clones*

To determine if MUC1 is a tumor antigen that can be recognized by cytotoxic T cells on the surface of MPM cells, we generated HLA-A*0201 restricted CD8+ T cell clones against MUC1(950-958) peptide. This peptide is presented in association with the HLA-A*0201 molecule to CD8+ T lymphocytes in numerous cancer types [20, 21]. PBMCs from HLA-A*0201+ healthy donors were stimulated three times by MUC1(950-958) peptide pulsed autologous DC, one week apart. Six days after the third stimulation, the presence of MUC1(950-958) specific T cells was determined by measuring IFN-γ producing cells in response to unpulsed or MUC1(950-958)-peptide pulsed TAP-deficient T2 cells. We found MUC1(950-958) specific CD8+ T cells in nine microcultures out of 80, with five cultures containing more than 10% of T cells specific for this epitope. By limiting dilution cultures, we then isolated two T cell clones: N5.14 and N32.10 from the two wells which contained the highest fraction of MUC1(950-958) specific T cells: well N5 and well N32 (fig.2A). To insure their clonality, we sequenced their CDR3 beta region and found that they both express a single TCR beta chain with a single CDR3 region (fig.2B). We then measured their reactivity against MUC1(950-958) peptide presented by HLA-A*0201+ T2 cells. We observed that the clone N5.14 (EC50=25.7nM +/- 4.4) has a higher avidity than clone N32.10

(EC50=866.7nM +/- 185.6), since it recognized about 34 times less peptide (fig.2C). We also observed that both clone responses to MUC1(950-958) peptide presented by T2 cells were inhibited by the presence of an anti-HLA-A*0201 mAb (clone BB7.2) in the co-culture (data not shown), confirming their HLA-A*0201 restriction.

*Recognition of HLA-A*0201+ MPM cells by HLA-A*0201 restricted MUC1(950-958) specific CD8+ T cell clones*

To determine if HLA-A*0201+ MPM cells are recognized by MUC1(950-958)/HLA-A*0201 specific T cell clones, we first co-cultured the two clones with either unpulsed or peptide pulsed T2 cells as control. We also analyzed and compared the clone responses when exposed to an HLA-A*0201- MPM cell line Meso13 or three HLA-A*0201+ MPM cell lines Meso35, Meso62 or Meso144 (fig.3A and 3B). As expected, the two clones responded to MUC1(950-958) peptide pulsed T2 cells and did not produce IFN-γ when co-cultured with the HLA-A*0201- Meso13 cell lines. However, only the clone N5.14, which displayed the best avidity toward the peptide (fig.2C), responded strongly to two HLA-A*0201+ MPM cell lines, Meso62 and Meso144, and weakly to the third HLA-A*0201+ one, Meso35 (fig.3A). The other clone N32.10, which exhibited a lower avidity, was not able to recognize the three HLA-A*0201+ MPM cell lines (fig.3B), except if the tumor cells were pulsed with peptides (data not shown). In this experiment, we also tested the response of the clones against the MPM cell lines treated 48h beforehand with IFN-γ which is known to increase the expression of molecules implicated in antigen presentation (CD54, CD58 and HLA class I) and also MUC1 expression (supplemental fig.1). IFN-γ treated HLA-A*0201+ MPM cell lines were better recognized by the clone N5.14 compared to untreated cell lines. However, the IFN-γ treatment did not lead to the recognition of the three HLA-A*0201+ MPM cell lines by the other clone, N32.10.

We extended this experiment to test the recognition by the two clones of all the HLA-A*0201+ MPM cell lines available in our laboratory. The T cell clone N5.14 responded to all HLA-A*0201+ MPM cell lines tested. This response was, in most cases, increased after the treatment of the tumor cell lines by IFN-γ, especially if the IFN-γ production by the clone was low in response to untreated tumor cell lines (fig.3C). This increase was significant ($p=0.0164$, Mann Whitney test) when results against all MPM cells lines were pooled together (fig.3D). In contrast, the clone N5.14 did not respond to the two HLA-A*0201- MPM cell lines, Meso4 and Meso13. The other CD8+ T cell clone, N32.10, failed to recognize any MPM cell lines (data not shown). These results suggest that the avidity of MUC1(950-958) specific T cells should be high enough to allow recognition of the naturally processed and presented peptide by MPM cells.

Then, to control the specificity of the clone N5.14, we performed two additional experiments. Firstly, we confirmed that MPM recognition by the clone is HLA-A*0201 restricted by adding an anti-HLA-A*0201 monoclonal antibody (clone BB7.2) in the T cell clone/MPM cell line co-culture (supplemental fig.2A). We observed a 60 to 70% inhibition of the clone IFN-γ response. Secondly, we assessed the clone N5.14 response against HCT116, a HLA-A*0201+ MUC1- colon cancer cell line (supplemental fig.2B). As expected, this tumor cell lines was not recognized by the clone N5.14, confirming its MUC1 specificity (supplemental fig.2C).

Cytolytic activity of CD8+ T cell clone N5.14 against MPM cell lines

We first observed evidences of HLA-A*0201+ MUC1+ MPM cell lines lysis by the clone N5.14 in a 50min intracellular Ca2+ level imaging experiment (fig.4A and supplemental fig.3). We observed that the clone N5.14 was not activated in the presence of the HLA-A*0201- MUC1+ MPM cell line Meso13. The clone moved from one tumor cell to

another, scanning for specific HLA/peptide complexes, and tumor cells stayed attached to the plastic. A few spikes of intracellular Ca2+ were observed in a few T cells, but were not sustained. In contrast, when the clone was cultured with the HLA-A*0201+ MUC1+ MPM cell line Meso144, majority of T cells stuck to tumor cells with a sustained intracellular Ca^{2+} increase. For the last 10min of the movie, some tumors cells recognized by the clone detached from the plastic and started dying (supplemental fig.3)

Finally, we confirmed that recognition of HLA-A*0201+ MPM cell lines by the clone N5.14 led to the lysis of tumor cells by a 4hr-^{51}Cr release assay. Thus, we co-cultured the clone N5.14 with two well-recognized HLA-A*0201+ MPM cell lines Meso62 or Meso144, or with one weakly recognized HLA-A*0201+ MPM cell line Meso35, or with the non-recognized HLA-A*0201- Meso13. We found that the clone N5.14 was able to kill the three HLA-A*0201+ MPM cell lines at levels correlated with its response measured by IFN-γ intracellular staining (fig.4B). As a control, HLA-A*0201- MPM cell line Meso13 was not killed by the clone.

*Glycosylation level of MUC1 does not affect recognition of HLA-A*0201+ MPM cells by HLA-A*0201 restricted MUC1(950-958) specific CD8+ T cell clones*

We did not find a correlation between the level of recognition of HLA-A*0201+ tumor cell lines by the clone and the surface staining of MUC1, CD54, CD58 and/or HLA class I by tumor cells (fig.1 and 3C). However, the staining with MUC1 specific mAb does not only reflect the quantity of surface MUC1, but also reflect the variable number of tandem repeated sequence present in MUC1 molecules. Furthermore, other parameters, such as MUC1 glycosylation status, could play a role in MUC1 presentation. Although several studies showed that recognition of MUC1 by T cells is increased when this antigen is hypoglycosylated [17, 19], our study showed that the glycosylation status of MUC1 did not seem to affect recognition of MPM cells. Indeed, some cell lines such as Meso62 with very low level of MUC1 hypoglycosylation were well recognized by the T cell clone. In contrast, Meso56 which exhibits the highest level of MUC1 hypoglycosylation, was one of the less recognized cell lines.

In addition, we used BGN (Benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside), a competitive inhibitor of O-glycosylation, to reduce glycosylation of MUC1 in MPM cells and then we tested whether this treatment increased the recognition of MPM cells by the T cell clone. As expected, when MPM cell lines were treated with BGN, MUC-1 glycosylation was severely impaired, since SM3 and VU-3-C6 stainings of MPM cell lines increased significantly close to levels observed with HMFG-1 (fig. 5A). However, T cell clone responses were similar on BGN treated and untreated cell lines (fig. 5B). This result confirms that the glycosylation status of MUC-1 does not affect presentation of MUC1(950-958)/HLA-A*0201 complexes on MPM cells and their recognition by CD8+ T cells.

Discussion

In this study, we have shown that all MPM cell lines express MUC1 with significant differences. The majority of MPM cell lines, such as Meso35 or Meso96, expresses at the cell surface normal MUC1 protein with low level of hypoglycosylation. Other MPM cell lines such as Meso13 or Meso56 express a more hypoglycosylated MUC1. Using two different MUC1(950-958)/HLA-A*0201 specific CD8+ T cell clones, we also demonstrated that all HLA-A*0201+ MPM cell lines of our study presented this epitope to the CD8+ T cell clone with the highest affinity, whatever the MUC1 glycosylation profile. In addition, MUC1 peptide presentation led to the lysis of HLA-A*0201+ MPM cell lines by this T cell clone. Combined, these results suggest that MUC1 may be a good candidate as a tumor antigen for the development of MPM immunotherapeutic treatments. However, the observation that the MUC1(950-958)/HLA-A*0201 specific CD8+ T cell clone with the lowest avidity does not recognize HLA-A*0201+ MPM cell lines, suggests that immunotherapeutic treatments of MPM should aim at inducing high avidity T cells against MUC1. Furthermore, monitoring of the T cell responses against MUC1 in such treatments should be performed against tumor cells, instead of target cells pulsed with high quantity of peptide.

Two other TAA expressed by MPM and able to induce CD8+ T cell response have been described summarily: mesothelin and WT1 [11, 12], since their recognition by peptide specific T cell lines were studied on a small number MPM cell lines (1 and 3 MPM cell lines, respectively). In our study, we thoroughly described MUC1 expression and glycosylation on 12 MPM cell lines and their recognition by a MUC1 specific CD8+ T cell clone. Nonetheless, these studies and ours suggest that at least three different TAA can be combined to target MPM in immunotherapeutic approaches, limiting the chance for tumor cells to escape immune system by the selection of antigen loss variants.

Our analysis of MUC1 expression and glycosylation shows that MUC1 is expressed by all the MPM cell lines analyzed with a variable MUC1 glycosylation profile from one MPM cell lines to another. MUC1 is often in a glycosylated form on the surface of MPM. This is characterized by absent or weak stainings by SM3 or VU-3C-6 mAb, which are induced or increased after treatment with BGN, a competitive inhibitor of glycosylation. However, a few MPM cell lines exhibit variable level of positive staining with SM3 or VU-3C-6 mAbs. Thus, some VNTR of MUC1 molecules at their surface are hypoglycosylated. Our results partly confirm previous observations from Creaney and collaborators who reported MUC1 expression in malignant mesothelioma [23], but we did not confirm the altered glycosylation of MUC1, since we observed hypoglycosylated profile of MUC1 molecules only for a few MPM cell lines.

We did not find a correlation between the staining level of surface MUC1 and the recognition of MPM cell lines by the MUC1 specific clone N5.14. For instance, MPM cell lines with low MUC1 surface staining, and no or weak hypoglycosylation, such as Meso47 or Meso62, were well recognized by the clone, whereas other MPM cell lines with high staining of surface MUC1, such as Meso56, were weakly recognized. It is not surprising that MUC1 staining does not correlate with level of recognition by the T cell clone, since these stainings do not reflect only MUC1 expression, but also the number of MUC1 VNTR. Furthermore, the presence of MUC1 on the surface of MPM cells does not account for the whole MUC1 expressed by tumor cells, but also depends on the turnover of MUC1 at the surface and its cleavage by sheddases such as TACE/ADAM17 and MT1-MMP [29, 30].

Additional parameters other than expression of MUC1 could modulate the presentation of MUC1(950-958) peptide to CD8+ T cells. We hypothesized that the glycosylation profile of MUC1 may be one of these parameters. Indeed, Hiltbold et al reported that glycosylation of long peptides, consisting of five MUC1 tandem-repeat regions, decreased the processing and the HLA-A1 restricted cross-presentation to CD8+ T cells by

DC of a nine amino acid peptide contained in this long peptide, [19]. Moreover, Hinoda and colleagues described an increased recognition of gastric tumor cells cultured with the O-glycosylation inhibitor BGN, by a HLA-unrestricted MUC1 specific CTL line [17]. It was clearly not the case in our study, since there was no correlation between MUC1 hypoglycosylation and T cell clone recognition. Furthermore, we performed several experiments to confirm the absence of influence of MUC1 glycosylation on the T cell clone response by treating the MPM cells with BGN, a competitive inhibitor of O-glycosylation. Treatment of MPM cell lines with BGN did not increase or induce their recognition by the T cell clone, suggesting that MUC1 glycosylation does not interfere with the HLA class I presentation of this epitope.

Several immunotherapeutic strategies have been developed to treat MPM. They exhibit high efficiency in mouse models and are currently being evaluated in phase I or phase II clinical trials. These strategies include injection to MPM patients of dendritic cells (DC) pulsed with autologous tumor cell lysate [10] of Type I interferon [31, 32], injection of IL-2 [33-35] or injection of CD40 agonist [36, 37]. All these strategies are mainly non antigen specific immunotherapies aimed at boosting anti-tumoral innate and specific immune responses, maturing antigen presenting cells or depleting regulatory T lymphocytes. With the identification of TAA expressed by MPM cells and recognized by T cells, such as MUC1, antigen specific immunotherapy could be designed to stimulate anti-tumor T lymphocyte responses. Such approaches targeting MUC1 have shown promising results in other malignancies like breast, prostate, lung, and ovarian cancer cancer [38-42]. For instance, in a pilot phase III immunotherapy study consisting of the injection of oxidized mannan-MUC1 to stage II breast cancer patients with no evidence of the disease, Apostolopoulos et al report that the vaccine prevents recurrence of the disease [38]. Thus, identification of tumor-associated antigens that are expressed by MPM, such as MUC1, and characterization of their recognition by cytotoxic T cells would be of great help in designing antigen specific immunotherapy to treat MPM.

Acknowledgements
This study was financed by INSERM, La ligue Régionale contre le Cancer, and the Nantes University Hospital.
We would like to thank Dr J. Le Pendu for his critical reading of the manuscript, D. Coulais from “Platform of Development and Clinical Transfer” and P. Hulin and S. Nedellec from MicroPICell - Cellular Imaging Core Facility of the Institut de Recherche Thérapeutique de Nantes for their technical assistance.

Conflict of interest
The authors declare no financial or commercial conflict of interest.

Reference
1. Robinson BW, Musk AW, Lake RA. Malignant mesothelioma. *Lancet* 2005: 366(9483): 397-408.
2. Scherpereel A, Astoul P, Baas P, Berghmans T, Clayson H, de Vuyst P, Dienemann H, Galateau-Salle F, Hennequin C, Hillerdal G, Le Pechoux C, Mutti L, Pairon JC, Stahel R, van Houtte P, van Meerbeeck J, Waller D, Weder W. Guidelines of the European Respiratory Society and the European Society of Thoracic Surgeons for the management of malignant pleural mesothelioma. *Eur Respir J*: 35(3): 479-495.
3. Gregoire M. What's the place of immunotherapy in malignant mesothelioma treatments? *Cell Adh Migr*: 4(1): 153-161.
4. Robinson BW, Robinson C, Lake RA. Localised spontaneous regression in mesothelioma -- possible immunological mechanism. *Lung Cancer* 2001: 32(2): 197-201.
5. Leigh RA, Webster I. Lymphocytic infiltration of pleural mesothelioma and its significance for survival. *S Afr Med J* 1982: 61(26): 1007-1009.
6. Anraku M, Cunningham KS, Yun Z, Tsao MS, Zhang L, Keshavjee S, Johnston MR, de Perrot M. Impact of tumor-infiltrating T cells on survival in patients with malignant pleural mesothelioma. *J Thorac Cardiovasc Surg* 2008: 135(4): 823-829.
7. Yamada N, Oizumi S, Kikuchi E, Shinagawa N, Konishi-Sakakibara J, Ishimine A, Aoe K, Gemba K, Kishimoto T, Torigoe T, Nishimura M. CD8+ tumor-infiltrating lymphocytes predict favorable prognosis in malignant pleural mesothelioma after resection. *Cancer Immunol Immunother*: 59(10): 1543-1549.
8. Gregoire M, Ligeza-Poisson C, Juge-Morineau N, Spisek R. Anti-cancer therapy using dendritic cells and apoptotic tumour cells: pre-clinical data in human mesothelioma and acute myeloid leukaemia. *Vaccine* 2003: 21(7-8): 791-794.

9. Ebstein F, Sapede C, Royer PJ, Marcq M, Ligeza-Poisson C, Barbieux I, Cellerin L, Dabouis G, Gregoire M. Cytotoxic T cell responses against mesothelioma by apoptotic cell-pulsed dendritic cells. *Am J Respir Crit Care Med* 2004: 169(12): 1322-1330.
10. Hegmans JP, Veltman JD, Lambers ME, de Vries IJ, Figdor CG, Hendriks RW, Hoogsteden HC, Lambrecht BN, Aerts JG. Consolidative dendritic cell-based immunotherapy elicits cytotoxicity against malignant mesothelioma. *Am J Respir Crit Care Med*: 181(12): 1383-1390.
11. Yokokawa J, Palena C, Arlen P, Hassan R, Ho M, Pastan I, Schlom J, Tsang KY. Identification of novel human CTL epitopes and their agonist epitopes of mesothelin. *Clin Cancer Res* 2005: 11(17): 6342-6351.
12. May RJ, Dao T, Pinilla-Ibarz J, Korontsvit T, Zakhaleva V, Zhang RH, Maslak P, Scheinberg DA. Peptide epitopes from the Wilms' tumor 1 oncoprotein stimulate CD4+ and CD8+ T cells that recognize and kill human malignant mesothelioma tumor cells. *Clin Cancer Res* 2007: 13(15 Pt 1): 4547-4555.
13. Vlad AM, Kettel JC, Alajez NM, Carlos CA, Finn OJ. MUC1 immunobiology: from discovery to clinical applications. *Adv Immunol* 2004: 82: 249-293.
14. Beatson RE, Taylor-Papadimitriou J, Burchell JM. MUC1 immunotherapy. *Immunotherapy*: 2(3): 305-327.
15. Barnd DL, Lan MS, Metzgar RS, Finn OJ. Specific, major histocompatibility complex-unrestricted recognition of tumor-associated mucins by human cytotoxic T cells. *Proc Natl Acad Sci U S A* 1989: 86(18): 7159-7163.
16. Noto H, Takahashi T, Makiguchi Y, Hayashi T, Hinoda Y, Imai K. Cytotoxic T lymphocytes derived from bone marrow mononuclear cells of multiple myeloma patients recognize an underglycosylated form of MUC1 mucin. *Int Immunol* 1997: 9(5): 791-798.
17. Hinoda Y, Takahashi T, Hayashi T, Suwa T, Makiguchi Y, Itoh F, Adachi M, Imai K. Enhancement of reactivity of anti-MUC1 core protein antibody and killing activity of anti-MUC1 cytotoxic T cells by deglycosylation of target tissues or cells. *J Gastroenterol* 1998: 33(2): 164-171.
18. Ho SB, Niehans GA, Lyftogt C, Yan PS, Cherwitz DL, Gum ET, Dahiya R, Kim YS. Heterogeneity of mucin gene expression in normal and neoplastic tissues. *Cancer Res* 1993: 53(3): 641-651.
19. Hiltbold EM, Alter MD, Ciborowski P, Finn OJ. Presentation of MUC1 tumor antigen by class I MHC and CTL function correlate with the glycosylation state of the protein taken Up by dendritic cells. *Cell Immunol* 1999: 194(2): 143-149.
20. Brossart P, Heinrich KS, Stuhler G, Behnke L, Reichardt VL, Stevanovic S, Muhm A, Rammensee HG, Kanz L, Brugger W. Identification of HLA-A2-restricted T-cell epitopes derived from the MUC1 tumor antigen for broadly applicable vaccine therapies. *Blood* 1999: 93(12): 4309-4317.
21. Brossart P, Schneider A, Dill P, Schammann T, Grunebach F, Wirths S, Kanz L, Buhring HJ, Brugger W. The epithelial tumor antigen MUC1 is expressed in hematological malignancies and is recognized by MUC1-specific cytotoxic T-lymphocytes. *Cancer Res* 2001: 61(18): 6846-6850.
22. Tsang KY, Palena C, Gulley J, Arlen P, Schlom J. A human cytotoxic T-lymphocyte epitope and its agonist epitope from the nonvariable number of tandem repeat sequence of MUC-1. *Clin Cancer Res* 2004: 10(6): 2139-2149.
23. Creaney J, Segal A, Sterrett G, Platten MA, Baker E, Murch AR, Nowak AK, Robinson BW, Millward MJ. Overexpression and altered glycosylation of MUC1 in malignant mesothelioma. *Br J Cancer* 2008: 98(9): 1562-1569.
24. Salter RD, Cresswell P. Impaired assembly and transport of HLA-A and -B antigens in a mutant TxB cell hybrid. *Embo J* 1986: 5(5): 943-949.

25. Fonteneau JF, Larsson M, Somersan S, Sanders C, Munz C, Kwok WW, Bhardwaj N, Jotereau F. Generation of high quantities of viral and tumor-specific human CD4+ and CD8+ T-cell clones using peptide pulsed mature dendritic cells. *J Immunol Methods* 2001: 258(1-2): 111-126.
26. Davodeau F, Difilippantonio M, Roldan E, Malissen M, Casanova JL, Couedel C, Morcet JF, Merkenschlager M, Nussenzweig A, Bonneville M, Malissen B. The tight interallelic positional coincidence that distinguishes T-cell receptor Jalpha usage does not result from homologous chromosomal pairing during ValphaJalpha rearrangement. *Embo J* 2001: 20(17): 4717-4729.
27. Arden B, Clark SP, Kabelitz D, Mak TW. Human T-cell receptor variable gene segment families. *Immunogenetics* 1995: 42(6): 455-500.
28. Cao Y, Karsten U. Binding patterns of 51 monoclonal antibodies to peptide and carbohydrate epitopes of the epithelial mucin (MUC1) on tissue sections of adenolymphomas of the parotid (Warthin's tumours): role of epitope masking by glycans. *Histochem Cell Biol* 2001: 115(4): 349-356.
29. Thathiah A, Carson DD. MT1-MMP mediates MUC1 shedding independent of TACE/ADAM17. *Biochem J* 2004: 382(Pt 1): 363-373.
30. Thathiah A, Blobel CP, Carson DD. Tumor necrosis factor-alpha converting enzyme/ADAM 17 mediates MUC1 shedding. *J Biol Chem* 2003: 278(5): 3386-3394.
31. Odaka M, Sterman DH, Wiewrodt R, Zhang Y, Kiefer M, Amin KM, Gao GP, Wilson JM, Barsoum J, Kaiser LR, Albelda SM. Eradication of intraperitoneal and distant tumor by adenovirus-mediated interferon-beta gene therapy is attributable to induction of systemic immunity. *Cancer Res* 2001: 61(16): 6201-6212.
32. Sterman DH, Recio A, Carroll RG, Gillespie CT, Haas A, Vachani A, Kapoor V, Sun J, Hodinka R, Brown JL, Corbley MJ, Parr M, Ho M, Pastan I, Machuzak M, Benedict W, Zhang XQ, Lord EM, Litzky LA, Heitjan DF, June CH, Kaiser LR, Vonderheide RH, Albelda SM, Kanther M. A phase I clinical trial of single-dose intrapleural IFN-beta gene transfer for malignant pleural mesothelioma and metastatic pleural effusions: high rate of antitumor immune responses. *Clin Cancer Res* 2007: 13(15 Pt 1): 4456-4466.
33. Ali G, Boldrini L, Lucchi M, Picchi A, Dell'Omodarme M, Prati MC, Mussi A, Corsi V, Fontanini G. Treatment with interleukin-2 in malignant pleural mesothelioma: immunological and angiogenetic assessment and prognostic impact. *Br J Cancer* 2009: 101(11): 1869-1875.
34. Astoul P, Picat-Joossen D, Viallat JR, Boutin C. Intrapleural administration of interleukin-2 for the treatment of patients with malignant pleural mesothelioma: a Phase II study. *Cancer* 1998: 83(10): 2099-2104.
35. Castagneto B, Zai S, Mutti L, Lazzaro A, Ridolfi R, Piccolini E, Ardizzoni A, Fumagalli L, Valsuani G, Botta M. Palliative and therapeutic activity of IL-2 immunotherapy in unresectable malignant pleural mesothelioma with pleural effusion: Results of a phase II study on 31 consecutive patients. *Lung Cancer* 2001: 31(2-3): 303-310.
36. Friedlander PL, Delaune CL, Abadie JM, Toups M, LaCour J, Marrero L, Zhong Q, Kolls JK. Efficacy of CD40 ligand gene therapy in malignant mesothelioma. *Am J Respir Cell Mol Biol* 2003: 29(3 Pt 1): 321-330.
37. Vonderheide RH, Flaherty KT, Khalil M, Stumacher MS, Bajor DL, Hutnick NA, Sullivan P, Mahany JJ, Gallagher M, Kramer A, Green SJ, O'Dwyer PJ, Running KL, Huhn RD, Antonia SJ. Clinical activity and immune modulation in cancer patients treated with CP-870,893, a novel CD40 agonist monoclonal antibody. *J Clin Oncol* 2007: 25(7): 876-883.
38. Apostolopoulos V, Pietersz GA, Tsibanis A, Tsikkinis A, Drakaki H, Loveland BE, Piddlesden SJ, Plebanski M, Pouniotis DS, Alexis MN, McKenzie IF, Vassilaros S. Pilot

phase III immunotherapy study in early-stage breast cancer patients using oxidized mannan-MUC1 [ISRCTN71711835]. *Breast Cancer Res* 2006: 8(3): R27.
39. Dreicer R, Stadler WM, Ahmann FR, Whiteside T, Bizouarne N, Acres B, Limacher JM, Squiban P, Pantuck A. MVA-MUC1-IL2 vaccine immunotherapy (TG4010) improves PSA doubling time in patients with prostate cancer with biochemical failure. *Invest New Drugs* 2009: 27(4): 379-386.
40. Loveland BE, Zhao A, White S, Gan H, Hamilton K, Xing PX, Pietersz GA, Apostolopoulos V, Vaughan H, Karanikas V, Kyriakou P, McKenzie IF, Mitchell PL. Mannan-MUC1-pulsed dendritic cell immunotherapy: a phase I trial in patients with adenocarcinoma. *Clin Cancer Res* 2006: 12(3 Pt 1): 869-877.
41. North SA, Graham K, Bodnar D, Venner P. A pilot study of the liposomal MUC1 vaccine BLP25 in prostate specific antigen failures after radical prostatectomy. *J Urol* 2006: 176(1): 91-95.
42. Ramlau R, Quoix E, Rolski J, Pless M, Lena H, Levy E, Krzakowski M, Hess D, Tartour E, Chenard MP, Limacher JM, Bizouarne N, Acres B, Halluard C, Velu T. A phase II study of Tg4010 (Mva-Muc1-Il2) in association with chemotherapy in patients with stage III/IV Non-small cell lung cancer. *J Thorac Oncol* 2008: 3(7): 735-744.

Figure legends

Figure 1: Expression of MUC1, CD54, CD58 and HLA class I molecules by MPM cell lines: MPM cells (Meso…) and breast cancer cells (MDA-MB231 and MCF-7) were stained with MUC-1 (clone HFMG-1, SM3 or VU-3-C6), CD54, CD58 or HLA-ABC specific mAb. Fluorescence was analyzed by flow cytometry. Grey histograms represent isotype control staining and white histogram MUC1, CD54, CD58 or HLA class I specific staining. Relative fluorescence intensity is shown on each histogram. This figure is representative of three experiments.

Figure 1

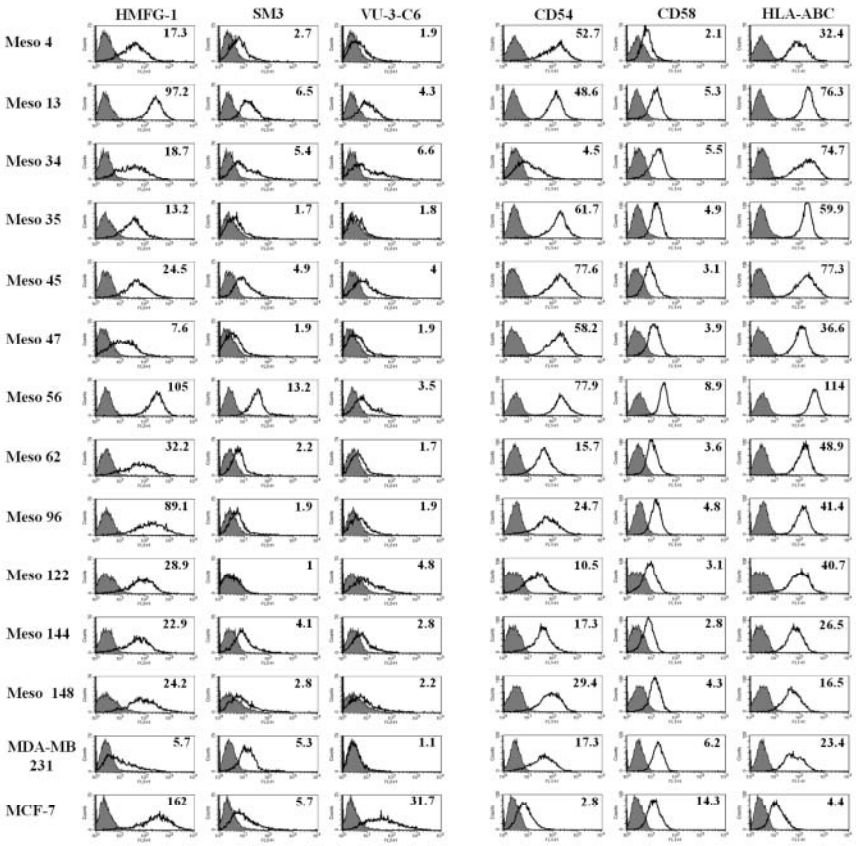


*Figure 2: Characterization of MUC1(950-958)/HLA-A*0201 specific T cell clones N5.14 and N32.10:* MUC1(950-958) pulsed HLA-A*0201+ mature DC were used to stimulate autologous T cells. Cultures were then restimulated weekly. (A) 6 days after the third stimulation, an aliquot of each T cell culture was exposed to unpulsed or MUC1(950-958) pulsed T2 cells. IFN-γ production by T cells was measured by intracytoplasmic IFN-γ staining

and flow cytometry analysis with a gate set on T cells. (B) MUC1(950-958)/HLA-A*0201 specific CD8+ T cell clones N5.14 and N32.10 were obtained by limiting dilution cultures of wells N5 and N32 respectively. Their TCR beta chain CDR3 regions were sequenced. (C) N5.14 and N32.10 CD8+ T cell clones were cultured with T2 cells pulsed with different concentrations of MUC1(950-958) peptides. IFN-γ production by T cell clones was measured by intracytoplasmic IFN-γ staining and flow cytometry analysis with a gate set on T cells. The figure is representative of three experiments.

Figure 2

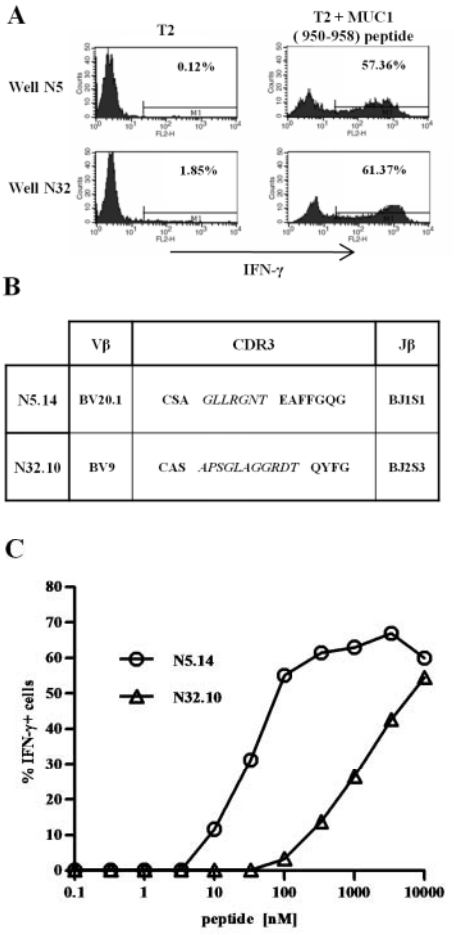


*Figure 3: Recognition of MPM tumor cells by MUC1(950-958)/HLA-A*0201 specific T cell clones N5.14 and N32.10:* (A) N5.14 and (B) N32.10 CD8+ T cell clones were cultured with HLA-A*0201- MPM cell line (Meso13), HLA-A*0201+ MPM cell lines (Meso62 or Meso144), T2 cells pulsed with 10μM of MUC1(950-958) peptide or Mesothelin(530-538) irrelevant peptide. IFN-γ production by T cell clones was measured by intracytoplasmic IFN-

γ staining and flow cytometry analysis with a gate set on T cells. These figures (3A and 3B) are issued from one experiment representative of three. (C) and (D) The CD8+ T cell clone N5.14 was cultured with MPM cell lines (Meso…) or breast cancer cell lines (MCF-7 or MDA-MB231). Tumor cells were treated or not with IFN-γ before coculture with T cell clone N5.14. IFN-γ production by T cell clones was measured by intracytoplasmic IFN-γ staining and flow cytometry with a gate set on T cells. (C) Histograms represent the mean percentage of cells secreting IFN-γ in response to each MPM cell lines obtained in three independent experiments. (D) Histograms represent the mean percentage of N5.14 T cell clone secreting IFN-γ in response to all HLA-A*0201+ MPM cell lines obtained in three independent experiments with a Mann Withney statistical analyses (*$p<0,05$).

Figure 3

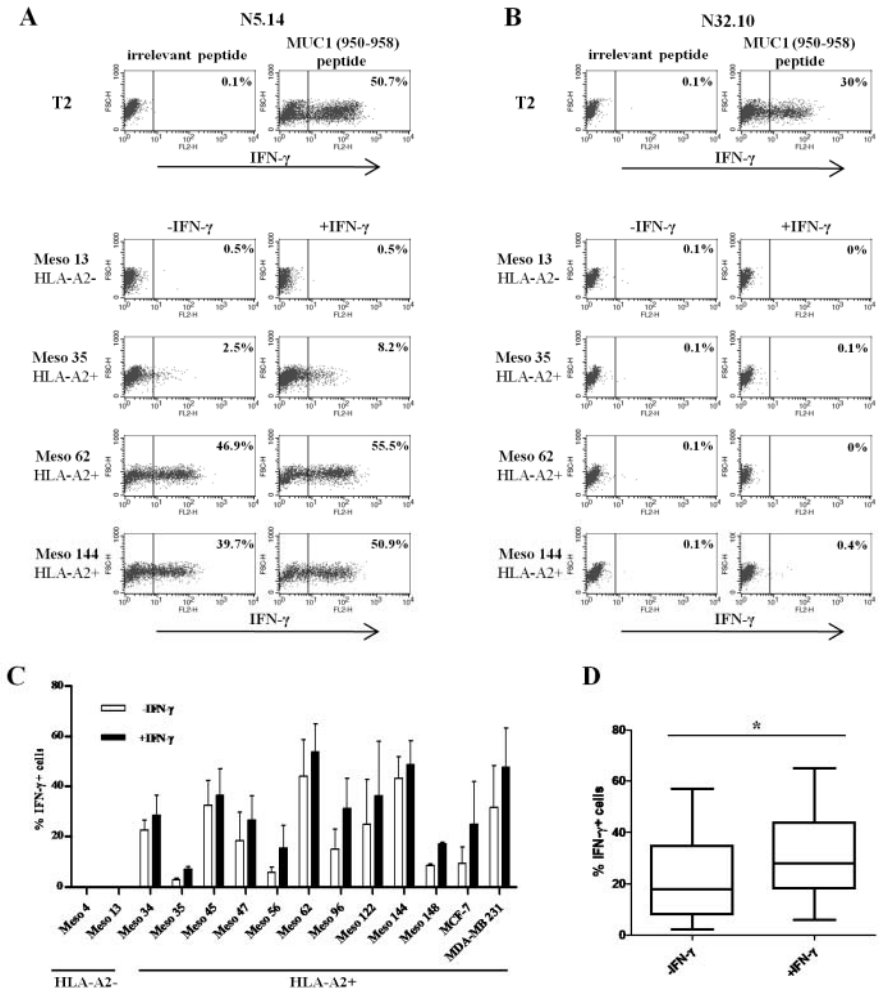


*Figure 4: intracellular Ca2+ and cytolytic response of the MUC1(950-958)/HLA-A*0201 specific T cell clones N5.14 in response to MPM cells:* (A) The clone N5.14 was pulsed with Fura2 Ca^{2+} sensitive probe and was cultured with MPM cell lines. Fluorescence was analysed by microscopy imaging. The pictures were taken after 8min of culture. Movie can be seen on supplemental fig.3 (B) Cytolytic activity of CD8+ T cell clone N5.14 against HLA-A2- MPM

cell lines Meso13 or HLA-A2+ MPM cell lines Meso35, meso62 or Meso144 was assessed by 4h ^{51}Cr release assay. This figure is issued of one experiment representative of three experiments. Each point represents mean of triplicate.

Figure 4

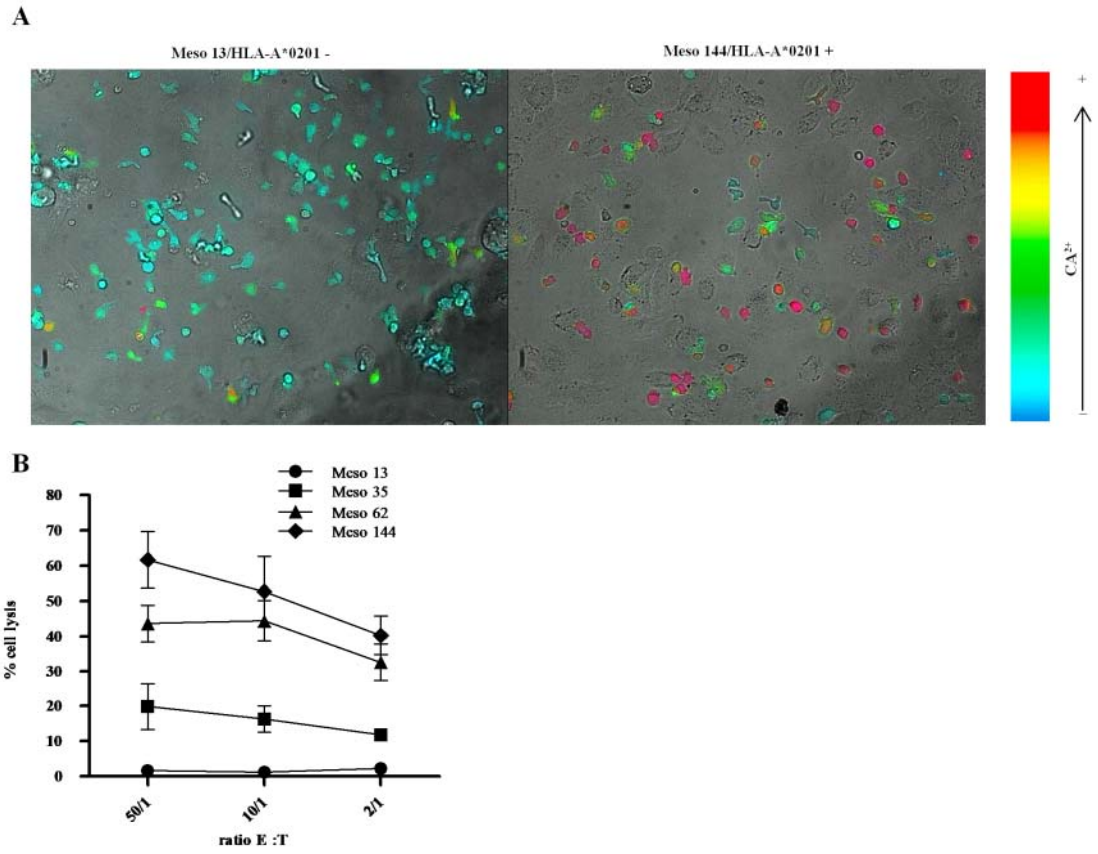
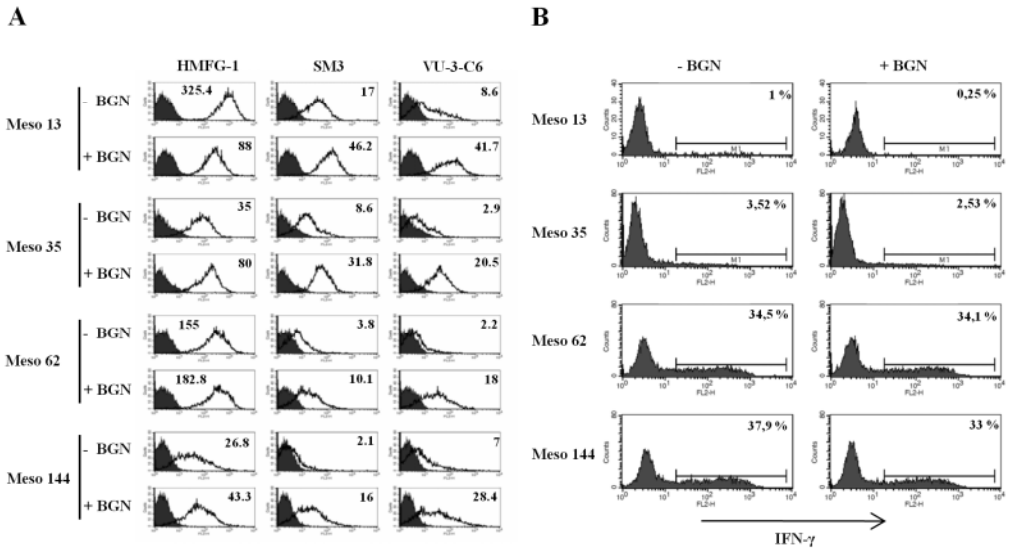


*Figure 5: Inhibition of MUC1 glycosylation by BGN restores staining with SM3 and VU-3-C6 monoclonal antibodies, but does not affect recognition of MPM cells by the MUC1(950-958)/HLA-A*0201 specific T cell clones N5.14:* (A) MPM cells were cultured for 48h with or without BGN, a competitive inhibitor of glycosylation, during the first 24h. MPM cells were then stained with MUC1 specific monoclonal antibodies HFMG-1, SM3 and VU-3-C6. Fluorescence was analyzed by flow cytometry. Relative fluorescence intensity is shown on each histogram. (B) The CD8+ T cell clone N5.14 was cultured with MPM cell lines, with or without prior BGN treatment. IFN-γ production by the T cell clone was measured by intracytoplasmic IFN-γ staining and flow cytometry with a gate set on T cells. This figure is representative of three experiments.

Figure 5


A
HMFG-1
SM3
VU-3-C6
Meso 13
- BGN
+ BGN
325.4
17
8.6
88
46.2
41.7
Meso 35
35
8.6
2.9
80
31.8
20.5
Meso 62
155
3.8
2.2
182.8
10.1
18
Meso 144
26.8
2.1
7
43.3
16
28.4
B
- BGN
+ BGN
Meso 13
1 %
0,25 %
Meso 35
3,52 %
2,53 %
Meso 62
34,5 %
34,1 %
Meso 144
37,9 %
33 %
IFN-γ

*Supplemental figure 3 : Movie of intracellular Ca^{2+} of the MUC1(950-958)/HLA-A*0201 specific T cell clones N5.14 in response to MPM cells:* (A) The clone N5.14 was pulsed with Fura2 Ca^{2+} sensitive probe and was cultured with MPM cell lines Meso13 (HLA-A*0201-) or Meso 144 (HLA-A*0201+). Fluorescence was analyzed by microscopy imaging with a picture taken every 15sec during 40min.