Eur Respir J 2011; 38: 1117–1126
DOI: 10.1183/09031936.00160210

Recognition of pleural mesothelioma by mucin-1(950–958)/human leukocyte antigen A*0201-specific CD8+ T-cells

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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 tumour antigenic targets. Mucin (MUC)1, which is expressed and recognised by cytotoxic T-cells in numerous cancer types, has not been investigated as a potential immune target in MPM. Thus, the objective of this study was to analyse MUC1 expression by MPM cells and to determine whether this antigen can be the target of cytotoxic CD8+ T-cells (cytotoxic T-lymphocytes (CTLs)).

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 for a MUC1 peptide (residues 950–958) presented by human leukocyte antigen (HLA)-A*0201 and studied its interferon-γ and cytotoxic response to MPM cell lines.

We found that all MPM cell lines expressed MUC1 protein at the cell surface with different glycosylation profiles. We also observed that HLA-A*0201+ MPM cell lines are recognised and lysed by a HLA-A*0201/MUC1(950–958)-specific CTL clone independently of the 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, immune response, mesothelioma, mucin 1

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Received:
Oct 12 2010
Accepted after revision:
April 08 2011
First published online:
May 03 2011

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

Clinical strategies developed as 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 correlation between the presence of lymphocytic infiltrate and better prognosis [4–7]. Furthermore, we previously showed, in pre-clinical studies, that cytotoxic T-lymphocytes (CTLs) can be generated against MPM [8, 9]. More recently, HEGMANS *et al.* [10] reported that injection of patients with dendritic cells (DCs) pulsed with an autologous tumour cell lysate is capable of inducing a CTL response against MPM.

The limit to the development of T-cell-based immunotherapeutic treatments of MPM is the lack of well characterised tumour-associated antigens (TAAs) recognised by T-cells. In the literature, recognition of MPM cells by TAA-specific CTLs has rarely been described and has not been analysed in details. YOKOKAWA *et al.* [11] showed that a CTL line specific for residues 547–556 of mesothelin and human leukocyte antigen (HLA)-A*0201 was able to lyse three mesothelin+ HLA-A*0201+ MPM cell lines, and similarly, a study by MAY *et al.* [12] showed that a CTL line specific to residues 122–140 of the Wilms' tumour 1 (WT1) oncoprotein and HLA-A*0201 was able to lyse one WT1+ HLA-A*0201+ MPM cell line.

This article has supplementary material available from www.erj.ersjournals.com

European Respiratory Journal
Print ISSN 0903-1936
Online ISSN 1399-3003

An additional TAA of interest is the mucin (MUC)1 antigen. This highly glycosylated type I transmembrane glycoprotein, with a variable number of 20-amino acid repeat sequences referred to as variable number tandem repeats (VNTRs), is now described as one of the most interesting targets for cancer immunotherapy [13, 14]. It was originally reported that VNTR sequences can be specifically recognised by CD8+ CTLs on the surface of numerous cancer cell types (breast cancer, pancreatic cancer and multiple myeloma) in a HLA class I-unrestricted fashion [15]. This recognition was dependent on a tumour-specific hypoglycosylation profile of MUC1 [16, 17], which 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 tumour cells by CTLs has also been demonstrated. One HLA-A1-restricted and several HLA-A*0201-restricted epitopes of MUC1, notably MUC1(950–958), which is recognised by CTLs on the surface of tumour cells, have been described [19–22] but not investigated in MPM.

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

MATERIALS AND METHODS

Tumour cell culture

Pleural effusions were collected by thoracocentesis, and diagnosis was established by immunohistochemical and immunocytochemical labelling. All patients gave signed, 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 characterised for several specific markers in our laboratory. The method of isolation of these cell lines is described elsewhere [24]. They all displayed an epithelioid phenotype. Human breast cancer cell lines MDA-MB231 (established by R. Cailleau, M.D. Anderson Hospital, Houston, TX, USA) and MCF-7 (established by B.J. Sugarman, Genentech Inc., San Francisco, CA, USA) were obtained respectively from D. Jäger (Klinik für Oncologie, Zürich, Switzerland) and from the American Type Culture Collection (Manassas, VA, USA). Cell lines were maintained in RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 U·mL^{-1} penicillin, 0.1 mg·mL^{-1} streptomycin and 2 mM L-glutamine (Sigma-Aldrich, Lyon, France). The T2 cell line (gift from T. Boon, Ludwig Institute for Cancer Research, Brussels, Belgium) is a HLA-A*0201+ human T-cell leukaemia/B-cell line hybrid defective for transporter associated with antigen processing (TAP)1 and TAP2, thus expressing empty HLA class I molecules at its surface that can be loaded with exogenous peptide [25]. Cells were cultured in a humidified atmosphere of 5% CO_2 at 37°C. In some experiments, tumour cell lines were cultured for 24 with 500 IU·mL^{-1} interferon (IFN)-γ (Abcys, Paris, France), or for 48 h with 5 mM benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside (BGN) (Sigma-Aldrich) during the first 24 h.

Antibodies and peptides

Phycoerythrin (PE)-conjugated mouse anti-human IFN-γ monoclonal antibody (mAb), mouse anti-human HLA-A2 mAb (clone BB7.2) and fluorescein isothiocyanate (FITC)-conjugated mouse immunoglobulin (Ig)G1k isotype control mAb were purchased from BD (Le Pont-De-Claix, France). Mouse anti-human MUC1 (clones HMFG-1 and SM3) mAbs were purchased from Abcam (Paris, France). Mouse anti-human MUC1 (clone VU-3C6) mAb was purchased from Santa Cruz Biotechnology (Tebu-bio, Le Perray en Yvelines, France). FITC-conjugated anti-human CD58 mAb, FITC-conjugated anti-human HLA-ABC mAb and PE-conjugated goat F(ab')$_2$ anti-mouse IgG (heavy and light chains (H+L)) were purchased from Beckman Coulter (Roissy, 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 ⩾95% pure.

T-cell priming

Blood from HLA-A*0201+ healthy donors was obtained from the Etablissement Français du Sang (Nantes, France). Induction of MUC1(950–958)/HLA-A*0201+ specific CD8+ T-cells was performed as we described previously but with minor modifications [26]. Briefly, T-cells were co-cultured in RPMI 1640 supplemented with 8% pooled human serum (pHS) produced locally with monocyte-derived DCs differentiated for 5 days with 1,000 IU·mL^{-1} granulocyte-macrophage colony-stimulating factor (Abcys) and 200 U·mL^{-1} interleukin (IL)-4 (Abcys), then matured for 24 h with 50 μg·mL^{-1} polyinosinic:polycytidylic acid (Sigma-Aldrich) and 20 ng·mL^{-1} tumour necrosis factor-α (Abcys), and pulsed for 2 h with 10 μM of MUC1(950–958) peptide. T-cell cultures were re-stimulated weekly with peptide-pulsed DCs in the presence of 10 U·mL^{-1} IL-2 (Proleukin; Chiron Corp., Emeryville, CA, USA) and 5 ng·mL^{-1} IL-7 (R&D Systems). 6 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 [26]. Briefly, T-cells were plated in U-bottom 96-well plates with irradiated (35 Gy) feeder cells (1×10^5 allogenic PBMCs and 1×10^4 Epstein–Barr virus-transformed B-cells per well), at concentrations of 10, 1 or 0.5 T-cells per well. The stimulatory medium consisted of RPMI 1640 containing 8% pHS, 150 U·mL^{-1} IL-2 and 1 μg·mL^{-1} phytohaemagglutinin L (Sigma-Aldrich). After 2 weeks, each clone was tested for peptide specificity. Specific clones were maintained in culture by periodic re-stimulation.

Complementarity-determining region 3β sequencing

RNA from 5×10^6 cells from each T-cell clone was extracted with the RNable reagent (Eurobio, Ulis, France) according to the manufacturer's instructions and dissolved in 15 μL water.

Reverse transcription, PCR amplification and sequencing were performed as described previously [27]. We followed the T-cell receptor (TCR) nomenclature established by ARDEN *et al.* [28].

Immunofluorescence and flow cytometry

For membrane staining, 1×10^5 cells were incubated at 4°C for 30 min with 1 μg·mL^{-1} specific or isotype-control mAb and washed. mAb dilution and washing were performed using PBS containing 0.1% bovine serum albumin (BSA) (Sigma-Aldrich). When unconjugated mAbs were used, a second incubation with PE-conjugated goat F(ab')$_2$ anti-mouse IgG (H+L) was performed. Fluorescence was analysed by flow cytometry (FacsCalibur; BD) using Cellquest software (BD). Relative fluorescence intensity (RFI) was calculated as the sample mean fluorescence divided by the isotype-control mean fluorescence.

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

Intracellular Ca^{2+} level video imaging

Measurement of intracellular Ca^{2+} levels was performed with CD8+ T-cell clone N5.14 loaded with 1 mM Fura-2/acetoxymethyl ester (Molecular Probes; Invitrogen, Villebon sur Yvette, France) for 1 h at room temperature in Hank's balanced salt solution (HBSS). T-cells were washed, resuspended in HBSS with 1% FCS and seeded on Lab-Tek glass chamber slides (Nunc, Naperville, IL, USA) coated with poly-L-lysine (Sigma-Aldrich). T-cells were co-cultured with tumour cells that were left to adhere to glass slides for 1 h at 37°C before addition of T-cells. Measurements of intracellular Ca^{2+} responses were performed at 37°C with a DMI 6000 B microscope (Leica Microsystems, Nanterre, France). Cells were illuminated every 15 s with a 300-W xenon lamp using 340/10- and 380/10-nm excitation filters. Emission at 510 nm was used for analysis of Ca^{2+} responses, and captured with a CoolSNAP HQ2 camera (Roper, Tucson, AZ, USA) and analysed with Metafluor 7.1 imaging software (Universal Imaging, Downington, PA, USA).

$^{51}Na_2CrO_4$ cytotoxicity assay

Tumour cell lines were incubated with $^{51}Na_2CrO_4$ (PerkinElmer, Boston, MA, USA) for 1 h at 37°C. 1×10^3 tumour cells (target) were then washed and co-cultured with a MUC1-specific CD8+ T-cell clone (effector) in a 96-well plate for 4 h at 37°C in triplicate. Effector/target ratios of 2/1, 10/1 and 50/1 were used. After a 4-h incubation at 37°C, 25 μL of each supernatant was collected and added to 100 μL scintillation liquid cocktail (OptiPhase Supermix; PerkinElmer) before liquid scintillation counting. The percentage of specific lysis was calculated as 100 × (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 that were lysed in media containing 1% Triton X-100 (Sigma-Aldrich).

RESULTS

MUC1 expression by MPM cells

We first performed a real-time PCR experiment to determine whether the *MUC1* gene was transcribed. Variable levels of *MUC1* transcript were detected in all MPM lines tested (data not shown). We then used flow cytometry to study the expression of MUC1 protein and 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 states of MUC1: HMFG-1, SM3 and VU-3-C6 [29]. Clone HMFG-1 recognises glycosylated and hypoglycosylated forms of MUC1, whereas clones SM3 and VU-3-C6 are specific for hypoglycosylated forms. Furthermore, these antibodies recognised the MUC1 VNTR motif, which is a 20-amino acid repeated sequence whose number varies from 20 to 125 repeats depending on 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 on the number of VNTRs 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 RFI ranging from 7.6 for Meso47 to 105.2 for Meso56. These staining levels are slightly lower than the one observed for MCF-7, a breast cancer cell line known to be recognised by MUC1-specific CD8+ T cells [20]. More staining heterogeneity was observed using the two mAbs 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. Together, these results suggest that all MPM cell lines express MUC1 with differences in the level and the type of glycosylation.

We also analysed the expression of molecules implicated in CD8+ T-cell activation, such as HLA-ABC (HLA class I), CD54 (intercellular adhesion molecule-1) and CD58 (lymphocyte function associated antigen-3) on MPM cells. All MPM cell lines 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-cell response.

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

To determine whether MUC1 is a tumour antigen that can be recognised by CTLs on the surface of MPM cells, we generated HLA-A*0201-restricted CD8+ T-cell clones against the MUC1(950–958) peptide. This peptide is presented in association with the HLA-A*0201 molecule to CD8+ T-cells in numerous cancer types [20, 21]. PBMCs from HLA-A*0201+

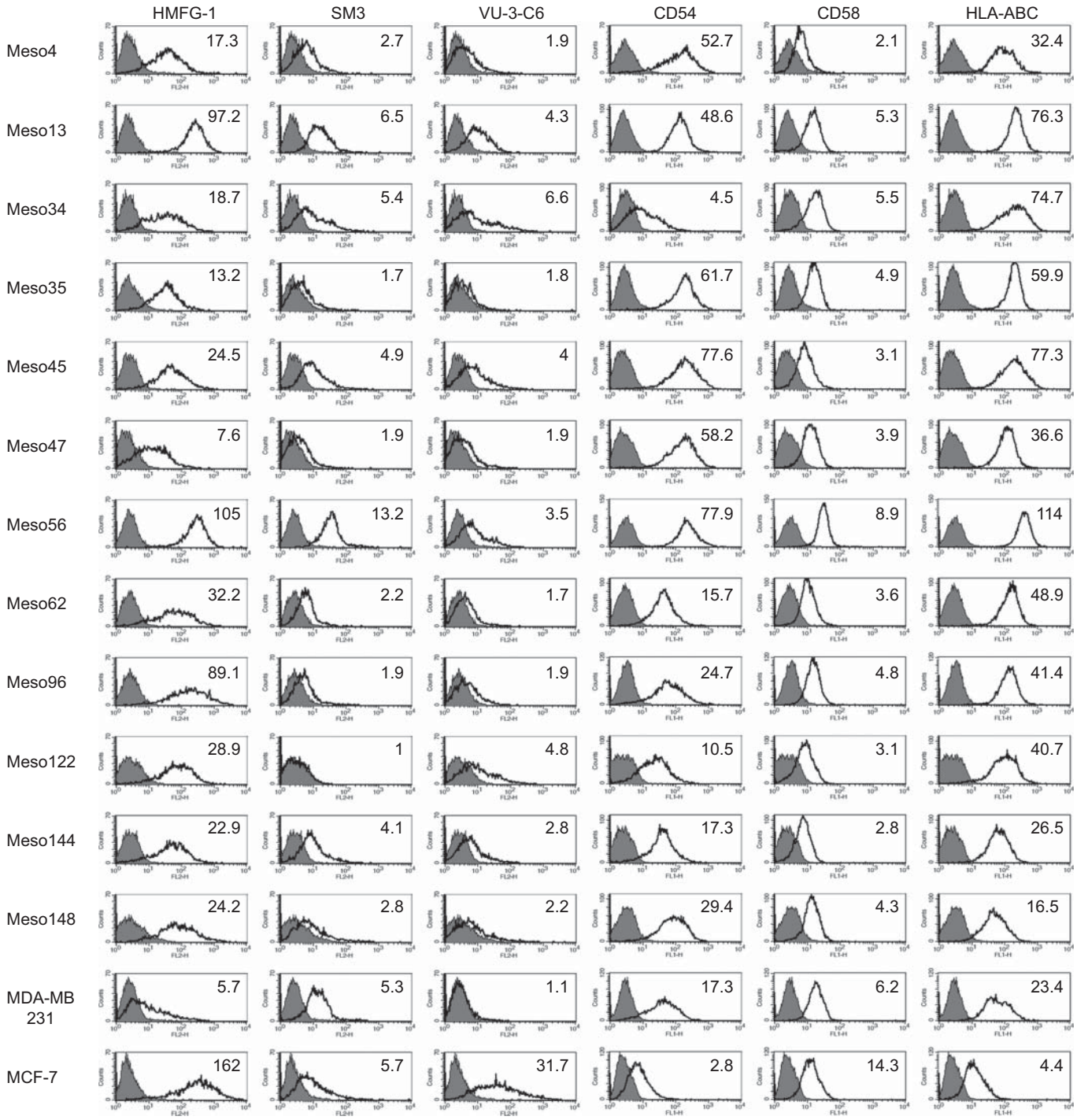

FIGURE 1. Expression of mucin (MUC)1, CD54, CD58 and human leukocyte antigen (HLA) class I molecules by malignant pleural mesothelioma (MPM) cell lines. MPM cells (Meso4, 13, 34, 35, 45, 47, 56, 62, 96, 122, 144 and 148) and breast cancer cells (MDA-MB231 and MCF-7) were stained with MUC1- (clones HFMG-1, SM3 and VU-3-C6), CD54-, CD58- or HLA-ABC-specific monoclonal antibodies. Fluorescence was analysed by flow cytometry. Grey histograms represent isotype control staining and white histograms MUC1-, CD54-, CD58- or HLA class I-specific staining. Relative fluorescence intensity is shown on each histogram. This figure is representative of three experiments.

healthy donors were stimulated three times with MUC1(950–958) peptide-pulsed autologous DCs, at 1-week intervals. 6 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 out of 80 microcultures, with five cultures containing >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 that contained the highest fraction of

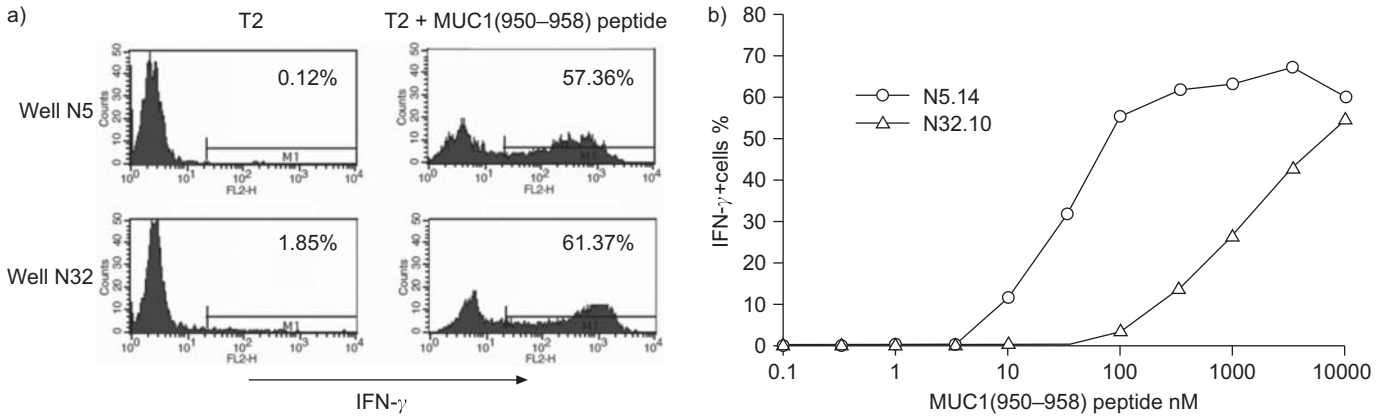

FIGURE 2. Characterisation of mucin (MUC)1(950–958)/human leukocyte antigen (HLA)-A*0201-specific T-cell clones N5.14 and N32.10. MUC1(950–958)-pulsed HLA-A*0201+ mature dendritic cells were used to stimulate autologous T-cells. Cultures were then re-stimulated weekly. a) 6 days after the third stimulation, an aliquot of each T-cell culture was exposed to unpulsed or MUC1(950–958)-pulsed T2 cells. Interferon (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) 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 gated for T-cells. The figure is representative of three experiments.

MUC1(950–958)-specific T-cells: wells N5 and N32 (fig. 2a). To ensure their clonality, we sequenced their complementarity-determining region (CDR)3β and found that they both expressed a single TCR β-chain with a single CDR3 region (table 1). We then measured their reactivity against MUC1(950–958) peptide presented by HLA-A*0201+ T2 cells. We observed that clone N5.14 (median effective concentration (EC_{50}) mean±SEM 25.7±4.4 nM) has a higher avidity than clone N32.10 (EC_{50} 866.7±185.6 nM), since it recognised ~34 times less peptide (fig. 2b). We also observed that both clones' 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 whether HLA-A*0201+ MPM cells are recognised 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 controls. We also analysed 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 and Meso144 (fig. 3a and b). 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 line. However, only 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+ line, Meso35 (fig. 3a). The other clone, N32.10, which exhibited a lower avidity, was not able to recognise the three HLA-A*0201+ MPM cell lines (fig. 3b), except if the tumour 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 48 h 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 (online supplementary fig. 1). IFN-γ-treated HLA-A*0201+ MPM cell lines were better recognised by clone N5.14 compared with 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.

TABLE 1 T-cell receptor (TCR) β-chain complementarity-determining region (CDR)3 sequences

Clone	Vβ	CDR3	Jβ
N5.14	BV20.1	CSA *GLLRGNT* EAFFGQG	BJ1S1
N32.10	BV9	CAS *APSGLAGGRDT* QYFG	BJ2S3

Mucin (MUC)1(950–958)/human leukocyte antigen (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 T-cell receptor β-chain complementarity-determining region CDR3 sequences were determined. Italics represent the hypervariable region. Vβ: variable region of TCRβ; Jβ: joining region of TCRβ.

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 tumour cell lines with IFN-γ, especially if the IFN-γ production by the clone was low in response to untreated tumour cell lines (fig. 3c). This increase was significant ($p=0.0164$ by Mann–Whitney U-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 recognise 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.

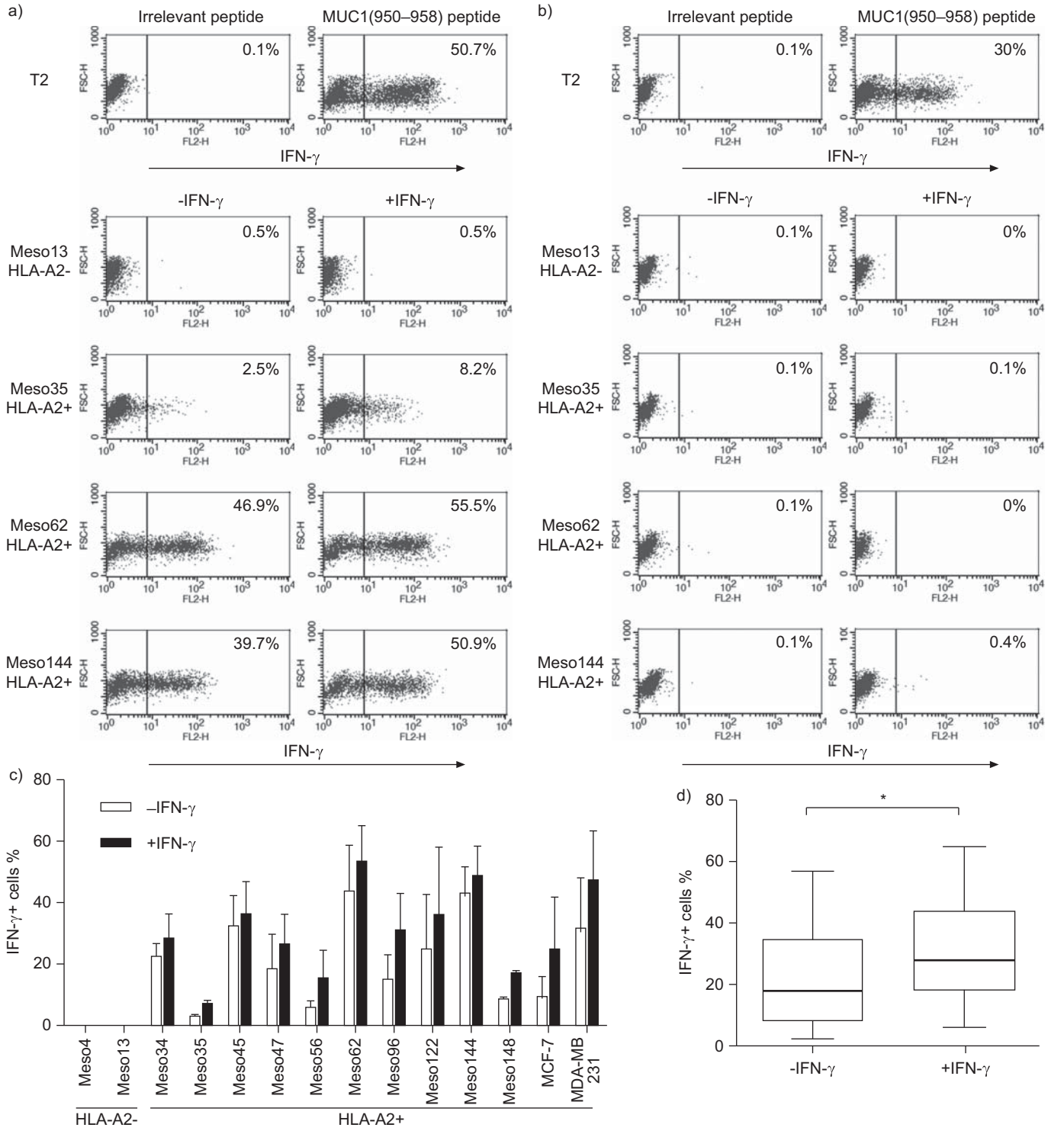

FIGURE 3. Recognition of malignant pleural mesothelial (MPM) tumour cells by mucin (MUC)1(950–958)/human leukocyte antigen (HLA)-A*0201-specific T-cell clones N5.14 and N32.10. a) N5.14 and b) N32.10 CD8+ T-cell clones were cultured with HLA-A*0201- (Meso13) or HLA-A*0201+ (Meso62 or Meso144) MPM cell lines, or T2 cells pulsed with 10 μM MUC1(950–958) peptide or an irrelevant mesothelin (530–538) peptide. Interferon (IFN)-γ production by T-cell clones was measured by intracytoplasmic IFN-γ staining and flow cytometry analysis gated for T-cells. Data are from one experiment that was representative of three. c and d) The CD8+ T-cell clone N5.14 was cultured with MPM cell lines (Meso4, 13, 34, 35, 45, 47, 56, 62, 96, 122, 144 and 148) or breast cancer cell lines (MCF-7 or MDA-MB231). Tumour cells were treated or not with IFN-γ before co-culture with T-cell clone N5.14. IFN-γ production by T-cell clones was measured by intracytoplasmic IFN-γ staining and flow cytometry gated for T-cells. c) Histograms represent the mean percentage of cells secreting IFN-γ in response to each MPM cell line obtained in three independent experiments. d) Box and whiskers plots of the percentage of N5.14 T-cells secreting IFN-γ in response to all HLA-A*0201+ MPM cell lines obtained in three independent experiments with a Mann–Witney statistical analysis. —: median; box: interquartile range; whiskers: range. *: $p<0.05$.

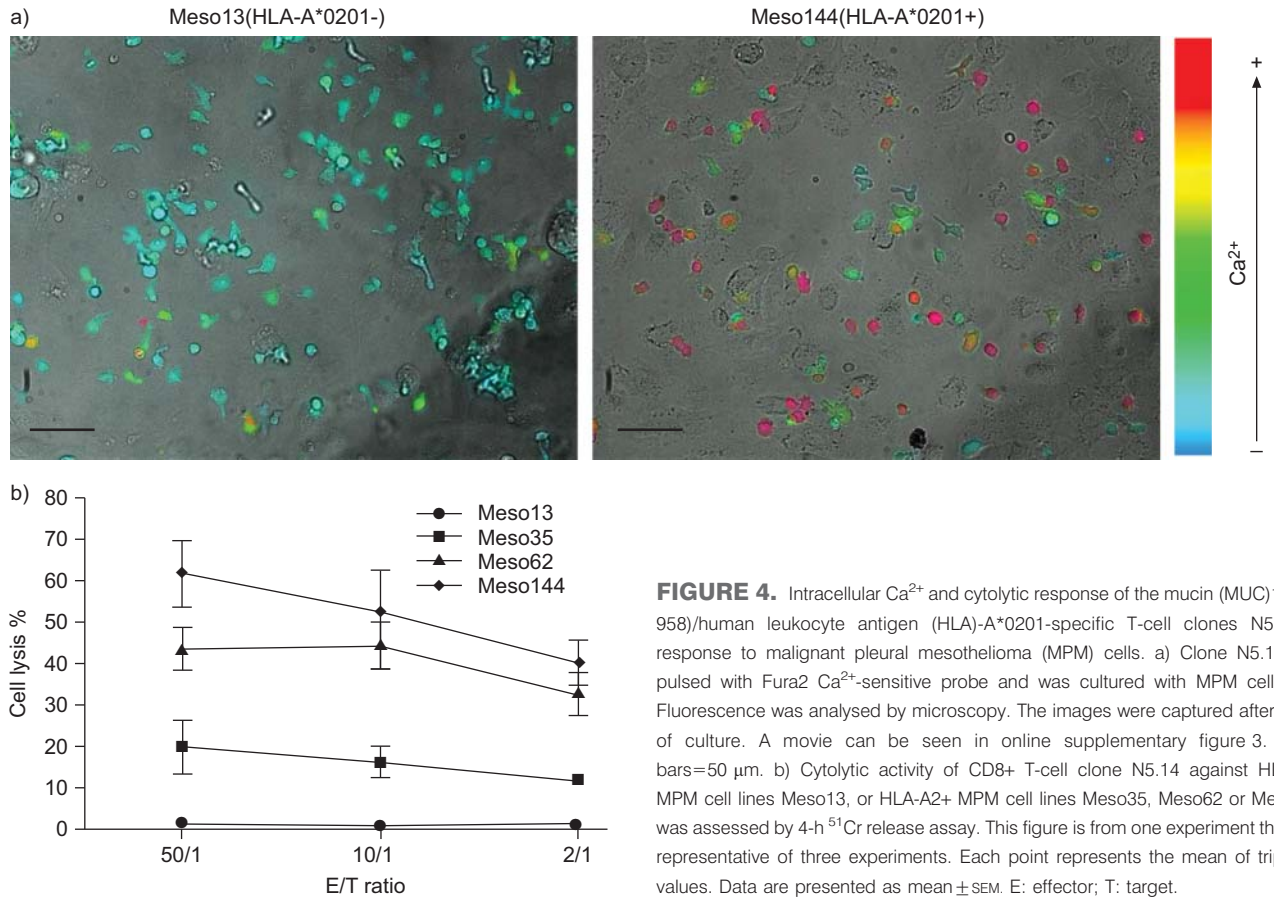

FIGURE 4. Intracellular Ca^{2+} and cytolytic response of the mucin (MUC)1(950–958)/human leukocyte antigen (HLA)-A*0201-specific T-cell clones N5.14 in response to malignant pleural mesothelioma (MPM) cells. a) Clone N5.14 was pulsed with Fura2 Ca^{2+}-sensitive probe and was cultured with MPM cell lines. Fluorescence was analysed by microscopy. The images were captured after 8 min of culture. A movie can be seen in online supplementary figure 3. Scale bars=50 μm. 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 4-h ^{51}Cr release assay. This figure is from one experiment that was representative of three experiments. Each point represents the mean of triplicate values. Data are presented as mean±SEM. E: effector; T: target.

Then, to control for the specificity of the clone N5.14, we performed two additional experiments. First, we confirmed that MPM recognition by the clone is HLA-A*0201-restricted by adding an anti-HLA-A*0201 mAb (clone BB7.2) in the T-cell clone/MPM cell line co-culture (online supplementary fig. 2a). We observed a 60–70% inhibition of the clone's IFN-γ response. Secondly, we assessed the response of clone N5.14 to HCT116, a HLA-A*0201+ MUC1- colon cancer cell line (online supplementary fig. 2b). As expected, this tumour cell line was not recognised by clone N5.14, confirming its MUC1 specificity (online supplementary fig. 2c).

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

We first observed evidence of HLA-A*0201+ MUC1+ MPM cell line lysis by clone N5.14 in a 50-min intracellular Ca^{2+} level imaging experiment (fig. 4a and online supplementary fig. 3). We observed that clone N5.14 was not activated in the presence of the HLA-A*0201- MUC1+ MPM cell line Meso13. The clone moved from one tumour cell to another, scanning for specific HLA/peptide complexes, and tumour cells remained attached to the plastic. A few spikes of intracellular Ca^{2+} 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, the majority of T-cells stuck to tumour cells with a sustained intracellular Ca^{2+} increase. For the last 10 min, some tumour cells recognised by the clone detached from the plastic and started dying (online supplementary fig. 3).

Finally, we confirmed that recognition of HLA-A*0201+ MPM cell lines by the clone N5.14 led to the lysis of tumour cells in a 4-h ^{51}Cr release assay. Thus, we co-cultured clone N5.14 with two well-recognised HLA-A*0201+ MPM cell lines, Meso62 or Meso144, or with one weakly recognised HLA-A*0201+ MPM cell line Meso35, or with the nonrecognised HLA-A*0201- line Meso13. We found that clone N5.14 was able to kill the three HLA-A*0201+ MPM cell lines at levels that correlated with its response measured by intracellular IFN-γ staining (fig. 4b). We used HLA-A*0201- MPM cell line Meso13 as control and it 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+ tumour cell lines by the clone, and the surface staining of MUC1, CD54, CD58 and/or HLA class I by tumour cells (figs 1 and 3c). However, staining with MUC1-specific mAbs not only reflects the quantity of surface MUC1, but also the VNTR sequences present in the 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 a very low level of MUC1 hypoglycosylation,

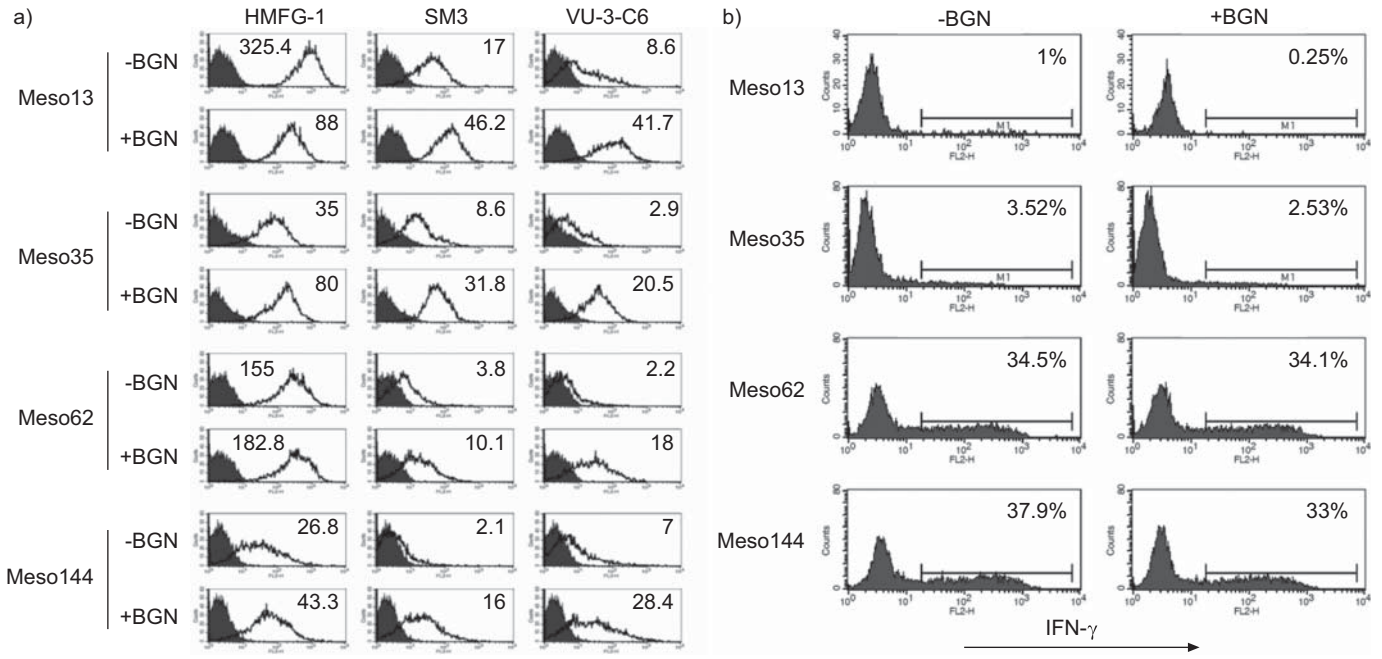

FIGURE 5. Inhibition of mucin (MUC)1 glycosylation by benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside (BGN) restores staining with SM3 and VU-3-C6 monoclonal antibodies (mAbs), but does not affect recognition of malignant pleural mesothelioma (MPM) cells by the MUC1(950–958)/human leukocyte antigen-A*0201-specific T-cell clone N5.14. a) MPM cells were cultured for 48 h with or without BGN, a competitive inhibitor of glycosylation, during the first 24 h. MPM cells were then stained with MUC1-specific mAbs HFMG-1, SM3 and VU-3-C6. Fluorescence was analysed 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. Interferon (IFN)-γ production by the T-cell clone was measured by intracytoplasmic IFN-γ staining and flow cytometry gated for T-cells. This figure is representative of three experiments.

were well recognised by the T-cell clone. In contrast, Meso56, which exhibits the highest level of MUC1 hypoglycosylation, was one of the less well-recognised cell lines.

In addition, we used BGN, a competitive inhibitor of *O*-glycosylation, to reduce glycosylation of MUC1 in MPM cells, and then 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, MUC1 glycosylation was severely impaired, as SM3 and VU-3-C6 staining of MPM cell lines increased significantly, close to levels observed with HMFG-1 (fig. 5a). However, T-cell clone responses were similar in BGN-treated and -untreated cell lines (fig. 5b). This result confirms that the glycosylation status of MUC1 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, express normal MUC1 protein with low level of hypoglycosylation at the cell surface. 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 in 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 tumour 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 recognise 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 tumour cells, instead of target cells pulsed with a high quantity of peptide.

Two other TAAs 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 was studied in a small number of MPM cell lines (one and three MPM cell lines, respectively). In our study, we thoroughly described MUC1 expression and glycosylation in 12 MPM cell lines and their recognition by a MUC1-specific CD8+ T-cell clone. Nonetheless, these studies and our own suggest that at least three different TAAs can be combined to target MPM in immunotherapeutic approaches, limiting the chance for tumour cells to escape the immune system by the selection of antigen loss variants.

Our analysis of MUC1 expression and glycosylation showed that MUC1 is expressed by all the MPM cell lines analysed with a variable MUC1 glycosylation profile from one MPM cell line to another. MUC1 is often in a glycosylated form on the surface of MPM cells. This is characterised by absent or weak staining by SM3 or VU-3-C6 mAbs, which are induced or increased after treatment with BGN, a competitive inhibitor of glycosylation. However, a few MPM cell lines exhibit a

variable level of positive staining with SM3 or VU-3-C6 mAbs. Thus, some VNTRs of MUC1 molecules are hypoglycosylated at their surface. Our results partly confirm previous observations by CREANEY *et al.* [23], who reported MUC1 expression in malignant mesothelioma, but we did not confirm the altered glycosylation of MUC1, since we observed a hypoglycosylated profile of MUC1 molecules for only 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 recognised by the clone, whereas other MPM cell lines with high staining of surface MUC1, such as Meso56, were weakly recognised. 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 VNTRs. Furthermore, the presence of MUC1 on the surface of MPM cells does not account for total MUC1 expressed by tumour cells, but also depends on the turnover of MUC1 at the surface and its cleavage by sheddases, such as tumour necrosis factor-α-converting enzyme/ADAM17 and membrane type 1 matrix metalloprotease [30, 31].

Additional parameters other than expression of MUC1 could modulate the presentation of MUC1(950–958) peptide to CD8+ T-cells. We hypothesised that the glycosylation profile of MUC1 may be one of these parameters. Indeed, HILTBOLD *et al.* [19] reported that glycosylation of long peptides, consisting of five MUC1 VNTRs, decreased the processing and the HLA-A1-restricted cross-presentation to CD8+ T-cells by DCs of a nine amino acid peptide contained in this long peptide. Moreover, HINODA *et al.* [17] described an increased recognition of gastric tumour cells cultured with the *O*-glycosylation inhibitor BGN, by a HLA-unrestricted, MUC1-specific CTL line. 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–II clinical trials. These strategies include injection of MPM patients with DCs pulsed with autologous tumour cell lysate [10] or type I IFN [32, 33], with IL-2 [34–36] or with a CD40 agonist [37, 38]. All these strategies are mainly nonantigen-specific immunotherapies aimed at boosting antitumour innate and specific immune responses, maturing antigen-presenting cells or depleting regulatory T-cells. With the identification of a TAA expressed by MPM cells and recognised by T-cells, such as MUC1, antigen-specific immunotherapy could be designed to stimulate antitumour T-cell responses. Such approaches targeting MUC1 have shown promising results in other malignancies, such as breast, prostate, lung and ovarian cancer [39–43]. For instance, in a pilot phase III immunotherapy study consisting of the injection of oxidised mannan–MUC1 to stage II breast cancer patients with no evidence of the disease, APOSTOLOPOULOS *et al.* [39] reported that this vaccine prevented recurrence of the disease. Thus, identification of TAAs that are expressed by MPM cells, such as MUC1, and characterisation of their recognition by CTLs would be of great help in designing antigen-specific immunotherapy to treat MPM.

SUPPORT STATEMENT

This study was financed by INSERM, La Ligue Inter-Régionale Contre le Cancer (comitees 44 and 79), the Nantes University Hospital (Nantes, France) and the ARSMESO44 association.

STATEMENT OF INTEREST

None declared.

ACKNOWLEDGEMENTS

We would like to thank J. Le Pendu (INSERM U892, Nantes, France) for his critical reading of the manuscript, D. Coulais (Platform of Development and Clinical Transfer, Hopital de Nantes, Nantes) and P. Hulin and S. Nedellec (MicroPICell, the Cellular Imaging Core Facility of the Institut de Recherche Thérapeutique de Nantes, Nantes) for their technical assistance.

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