Mucin-1 is expressed on dendritic cells, both in vitro and in vivo

Silvie Cloosen, Marco Thio, Ariane Vanclée, Ellen B. M. van Leeuwen, Birgit L. M. G. Senden-Gijsbers, Ellis B. H. Oving, Wilfred T. V. Germeraad and Gerard M. J. Bos

Department of Internal Medicine, Division of Hemato-Oncology, University Hospital Maastricht, PO Box 5800, 6202 AZ Maastricht, The Netherlands

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Abstract

Dendritic cells (DCs) are the best professional antigen-presenting cells to stimulate cytotoxic as well as T helper cells and are therefore appropriate candidates for establishing immunotherapy. The concept of our vaccination program is to introduce the tumor-associated antigen mucin-1 (MUC1) into DCs. Analysis of immature and mature DCs—before transducing the antigen MUC1—already demonstrated expression of MUC1 on in vitro monocyte-derived DCs upon maturation. Different culture methods as well as maturation cocktails showed similar results concerning the upregulation of MUC1 expression. Furthermore, we studied the expression of MUC1 on DCs in vivo. No MUC1 expression was found on blood DCs, or on thymic or tonsil DCs. On the other hand, synovial fluid from patients with arthritis contained DCs that were found to express MUC1. This study shows for the first time that the tumor-associated antigen MUC1 is expressed on in vivo DCs. We further show that MUC1 is also expressed on in vitro cultured bone marrow-derived DCs of human MUC1 transgenic mice, supporting the relevance of this mouse model to the human situation. The observation that MUC1 is present on in vivo DCs suggests a functional role, but this physiological function remains to be elucidated.

Introduction

Today, traditional cancer treating methods (surgery, radiotherapy and chemotherapy) are far from sufficient and new strategies including immunotherapy are needed more than ever. Active vaccination, using tumor antigen loaded DCs, the best antigen-presenting cells to be used for inducing cytotoxic T cell as well as T helper cell responses, became an attractive option. In various studies, encouraging clinical effects have been observed (1–3), although with limited success (4,5). This could be due to the state of the patients, who are frequently in the final stage of their disease, the type of tumor that is studied, the type of DC used regarding its maturation, differentiation or activation, or the choice of tumor antigens. Therefore, optimization of these studies remains to be further investigated. Fortunately, all DC vaccines are being well tolerated by most patients (5,6), with the major side effects being vitiligo (5), temporal erythema at the injection site (7) or a low fever (8).

Among the high number of possible tumor antigens, we have chosen to work with MUC1. This is a transmembrane glycoprotein expressed at the apical cell surface of normal glandular epithelium such as breast, ovari,

Correspondence to: S. Cloosen; E-mail: S.Cloosen@immuno.unimaas.nl

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The concept of our vaccination program is to transduce DCs with MUC1 via a modified adenoviral vector (31) to realise a better transduction, a higher and prolonged expression resulting in continuous peptide presentation, expected to be superior to peptide and protein loading of DCs. Before transducing DCs with MUC1 cDNA, we analysed immature and mature DCs for MUC1 expression. Surprisingly, we observed that DCs show MUC1 upregulation upon maturation of monocyte-derived DCs (MoDCs). During the course of our investigation, a similar observation has been published by Wykes et al. (32). In this paper we describe that MUC1 is present on MoDCs irrespective of the culture and maturation method. Moreover, MUC1 is also present on in vivo DCs, in synovial fluid, suggesting a physiological role and not merely an in vitro phenomenon. In addition, MUC1 could also be demonstrated on bone marrow-derived DCs of human MUC1 transgenic (hMUC1 Tg) mice. The presence of MUC1 on mature DCs is relevant for immunotherapy using MUC1 as a tumor associated antigen of choice. The physiological role of MUC1 on DCs in vivo remains to be elucidated.

Methods
Antibodies and cell lines
Hybridomas HMF1G1 and HMF2G2 produce anti-human MUC1 mouse mAbs specific for glycosylated as well as underglycosylated MUC1 and were kindly provided by Dr J. Taylor-Papadimitriou (Guy’s Hospital, London, UK). Hybridoma 214D4 produces anti-human MUC1 mAbs specific for glycosylated as well as underglycosylated MUC1 and was kindly provided by Dr J. Hilkens (The Netherlands Cancer Institute, Amsterdam, The Netherlands). DF3 is a mAb specific for glycosylated as well as underglycosylated MUC1, kindly provided by Dr S. von Mensdorff-Pouilly (VU Medical Center, Amsterdam, The Netherlands). The mAbs 214D4 and DF3 were biotinylated at our department and detected by peridinin chlorophyll-a protein (PerCP)-conjugated streptavidin (BD Biosciences/Pharmingen, San Diego, CA). Unconjugated as well as biotinylated isotype control mouse immunoglobulin G1 (IgG1) were purchased from BD Biosciences/Pharmingen. Secondary rabbit anti-mouse R-phycocerythrin-Cy5 (R-PE-Cy5)-conjugated antibodies were purchased from Dako Cytomation (Glostrup, Denmark). R-PE-conjugated anti-human HLA-DR, CD14, CD33, CD123, CD40, CD1a, FITC conjugated anti-human lineage cocktail (Lin; CD3, CD14, CD16, CD19, CD20, CD56), CD86, CD83, CD80 and allophycocyanin (APC)-conjugated anti-human CD11c, CD11b, HLA-DR were purchased from BD Biosciences/Pharmingen. Rat anti-mouse CD16/CD32 (Fc block), FITC-conjugated rat anti-mouse IA-IE, R-PE-conjugated hamster anti-mouse CD11c, biotinylated mouse IgG1, x and PerCP conjugated streptavidin were purchased from BD Biosciences/Pharmingen.

The cell line secreting recombinant murine GM-CSF was a kind gift of Dr M. M. Lutz (University of Erlangen, Germany). This is an Ag 8653 myeloma cell line transfected with murine GM-CSF cDNA, produced by Dr B. Stockinger (National Institute for Medical Research, Mill Hill, London, UK). The cells were cultured in RPMI 1640 medium (Life Technologies, Breda, The Netherlands) with 10% fetal calf serum (FCS; Greiner Bio-One GmbH, Frickenhausen, Germany) and 1% penicillin/streptomycin (Life Technologies). When the cells reached a high density, the supernatant was collected and filtered to remove remaining cells. Supernatant was stored at 4°C and used at a concentration of 10% in DC cultures. The breast carcinoma cell line ZR75-1, expressing MUC1 at high levels, was grown in Iscove’s MDM supplemented with 10% FCS (Greiner Bio-One) and used as a positive control for MUC1 expression in FACS analysis.

Human DC culture
Buffy coats from normal healthy donors were obtained from the blood bank (Sanquin, Maastricht, The Netherlands). Peripheral blood monocytes were isolated by ficoll gradient (lymphoprep d = 1.077, Axis-Shield, Oslo, Norway) centrifugation for 20 min at 640 g at room temperature. The light-density fraction was collected and washed four times using phosphate-buffered saline (PBS; Life Tech.) containing 2 mM EDTA (Merck, Darmstadt, Germany). T cells were removed by sheep red blood cell (Animal facility, University Maastricht, The Netherlands) rosetting, followed by ficoll gradient (Axis- Shield) centrifugation. Sheep red blood cells present in the T cell pellet were lysed using 160 mM ammonium chloride (Merck), T cells were washed once in PBS (Life Technologies) and frozen for later use. The light-density fraction containing monocytes and B cells was collected and left to adhere in tissue culture flasks (Corning Costar, Cambridge, UK) for 1.5 h at 37°C and 5% CO₂. The non-adherent fraction containing the B cells was removed and frozen for later use. The adherent fraction was further cultured at 10⁶ cells/ml in RPMI 1640 (Life Technologies)/1% autologous plasma/1% penicillin/streptomycin (Life Technologies)/1% asparagine/arginine/glutamine (Life Technologies) for 6 days, adding 500 U/ml IL-4 (Sanquin, Amsterdam, The Netherlands) and 20 ng/ml GM-CSF (Immune, Seattle, WA) on days 0, 2 and 4 by gently replacing the upper half of the medium. On day 6, immature DCs were further matured for 48 h using a cytokine cocktail consisting of 1000 U/ml IL-6 (Biosource, Etten-Leur, The Netherlands), 200 U/ml TNF-α (Biosource, Etten-Leur, The Netherlands), 10 ng/ml IL-1β (BD Biosciences/Pharmingen) and 10 μg/ml PGE₂ (Sigma) in the continuous presence of IL-4 (Sanquin) and GM-CSF (ImmuneX). We also obtained immature MoDCs from PID (Paris, France) either fresh or frozen in vials (33). Both were cultured overnight in AIM-V medium (Life Technologies)/1% penicillin/streptomycin (Life Technologies) in the continuous presence of 50 ng/ml IL-13 (Sanofi-Synthelabo, kindly provided by IDM) and 500 U/ml GM-CSF (ImmuneX). DCs were matured for 48 h by adding 1 μg/ml Ribonuclyl (Pierre Fabre medicament production, Boulogne, France, kindly provided by IDM) and 500 U/ml IFN-γ (Strathmann Biotec AG, Hannover, Germany, kindly provided by IDM).

Mice and DC culture
MUC1 Tg breeder mice on a C57Bl/6 background were kindly provided by Dr S. Gendler (Mayo Clinic Scottsdale, AZ). Tg mice were bred with C57Bl/6 mice obtained from Charles River (Maastricht, The Netherlands). Animals were housed at our animal facilities where they were provided with normal rodent chow and allowed water ad libitum. MUC1 Tg mice and
sex matched wild-type littermates were used for experiments at 11–18 weeks of age. Bone marrow-derived DCs were generated from the femurs and tibia of the mice. The bone marrow was flushed out using RPMI 1640 (Life Technologies) and subsequently passed through wire mesh screens to obtain a single cell suspension. Fresh bone marrow cells were seeded at 2 × 10^6 cells in 10 ml RPMI 1640 (Life Technologies) containing 2 mM glutamine (Life Technologies) and supplemented with 50 µM β-mercaptoethanol (Merck)/10% FCS (Greiner Bio-One)/1% penicillin/streptomycin (Life Technologies)/2 ng/ml murine recombinant IL-4 (Pepro Tech, Rocky Hill, NJ) and 10% supernatant of the GM-CSF producing cell line. Another 10 ml of medium, including growth factors as described above, was added to each dish on day 3. On days 6 and 8, half of the medium was replaced by adding 10 ml of fresh medium to each dish. Maturation was induced by adding 1 µg/ml lipopolysaccharide (LPS) (Difco Brunschwig, Amsterdam, The Netherlands) for the last 24 h.

**In vivo DC isolation and characterization**

**Blood**

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood from healthy donors by lymphoprep (Axis-Shield) density centrifugation. Two populations of DCs can be defined in blood by FACS analysis based on the level of CD33 expression, CD33^hi and CD33^int in combination with the absence of lineage markers (Lin^−) (34,35).

**Synovial fluid**

Synovial fluid was obtained from patients visiting the Rheumatology clinic of our hospital and suffering from arthritis. Mononuclear cells were obtained by lymphoprep (Axis-Shield) density centrifugation. Dendritic cells were phenotypically defined as Lin^−, CD33^hi, HLA-DR^+ population. Within this population, CD123+ plasma cells and CD11c+ myeloid DCs, as well as CD11b+ myeloid DCs, were defined by FACS analysis based on the level of CD33 expression, CD33^hi and CD33^int in combination with the absence of lineage markers (Lin^−) (34,35).

**Thymus and tonsil**

Thymus were obtained from children between 2 days and 5 years old undergoing cardiac surgery (University Hospital of Cologne, Germany). Tonsils were obtained from young children undergoing regular tonsillectomy (Academic Hospital Maastricht, The Netherlands). The organs were cut into small pieces with scissors and digested for 30 min at 37°C with Collagenase type IV (Sigma, Zwijndrecht, The Netherlands) resulting in a 351 bp product on a 1% agarose gel.

**Flow cytometry**

Human cells were incubated with mAbs DF3, 214D4, HMFG1 or HMFG2 for 60 min on ice, followed by incubation with R-PE-Cy5-conjugated rabbit anti-mouse immunoglobulins for another 30 min on ice. Empty binding sites of rabbit anti-mouse immunoglobulins were blocked for 10 min by incubation with mouse serum (Dako Cytomation) on ice. Finally, cells were counterstained with appropriate cell markers labelled with a fluorochrome for another 30 min incubation on ice. Biotinylated anti-MUC1 mAbs were detected using PerCP-conjugated streptavidin. Cells were fixed in 1% paraformaldehyde and analysed on a FACSort cytometer (BD, San Diego, CA). Dead cells were excluded based on their forward/side scatter properties.

To analyse *in vitro* bone marrow-derived DCs of the hMUC1 Tg mice, nonadherent cells were removed from the culture dishes, centrifuged and resuspended in PBS (Life Technologies) supplemented with 0.1% FCS (Greiner Bio-One) and 0.02% sodium azide (Merck). After preincubation with mouse Fc block for 10 min, cells were incubated with FITC-conjugated rat anti-mouse IA-IE, R-PE-conjugated hamster anti-mouse CD11c, and biotinylated mouse anti-human DF3 or an isotype control for 30 min on ice. Subsequently cells were washed and incubated for another 15 min with Per-CP-conjugated streptavidin on ice. Analysis was performed on a FACSort cytometer (BD). Again, dead cells were excluded based on their forward/side scatter properties. DCs are present in the larger FSC/SSC gate and express CD11c. Distinction of immature and mature DCs was made by low expression of MHc class II on immature DCs in contrast to mature DCs that express high levels.

**RT-PCR**

Total RNA was isolated using QIAshredder and RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA was generated using a SuperScript™ One-Step™ RT-PCR kit (Life Technologies) according to the manufacturer's instructions. The cDNA was subsequently amplified using MUC1-specific primers 5’-GTACCATCAATGTCACGAC-3’ and 5’-CTACGATCGTACTGTTAGG-3’ (Eurogentec, Maastricht, The Netherlands) resulting in a 351 bp product on a 1% agarose gel.

**Mixed lymphocyte reaction**

Mature DCs were obtained as described above. Subsequently, DCs were incubated with mAb DF3 or isotype control (both at a final concentration of 10 µg/ml) on ice and at 37°C for 1 h. Next, detection of cell surface MUC1 was carried out with rabbit anti-mouse R-PE-Cy5 by FACS analysis. DCs expressing cell surface MUC1 (isotype control incubated at 37°C) or not (DF3 incubated at 37°C) were used in a mixed lymphocyte reaction (MLR). In each well of a 96-well plate, 10^5 stimulator cells were added to 10^5 autologous, 10^5 allogeneic T cells and 10^5 naive PBMC from cord blood, and incubated for 4 days at 37°C. During the incubation period, we continued adding each day either mAb DF3 (10 µg/ml) or isotype (10 µg/ml) control to the mature DCs. A proportion of the mature DCs in the MLR was analysed for MUC1 expression by FACS analysis as described above, just before thymidine addition. The incubation with the antibodies was carried out on ice. Sixteen hours after addition of thymidine, proliferation of T cells was measured using a β-counter.
Immunohistochemistry

Thymi from children undergoing cardiac surgery were embedded in OCT compound (Sakura Finetek, Zoeterwoude, The Netherlands), snap frozen and stored at –80°C until analysis. Five-micrometer frozen sections were cut and fixed in acetone (Merck) followed by 1 h incubation with anti-CD11c (Dako, Glostrup, Denmark) in PBS/1% BSA/0.1% Tween. Subsequently, sections were washed with PBS (Life Technologies), incubated with goat-anti-mouse Envision HRP (Dako) for 30 min and developed with 3-amino-9-ethylcarbazole (AEC; Sigma). After rinsing with demi-water, sections were incubated for 20 min with 2% normal mouse serum (Dako) in PBS/1% BSA/0.1% Tween, followed by biotinylated anti-MUC1 (214D4) for 1 h in PBS/1% BSA/0.1% Tween. Final development was carried out with ABC-AP (Dako) for 30 min and BCIP-NBT (Dako) for 5 min. Sections were sealed with Imsol (Klinipath, Duiven, The Netherlands). All incubations were carried out at room temperature and staining reagents were all used at the proper dilutions.

Results

In vitro MoDCs show MUC1 upregulation upon activation

Before transducing DCs with MUC1 cDNA, we analysed fresh CD14+ monocytes in blood and after isolation, as well as cultured immature and mature DCs for MUC1 expression. MoDCs were obtained by in vitro culture of CD14+ monocytes in the presence of IL-4 and GM-CSF for 6 days, followed by maturation for an additional 2 days using the maturation cocktail IL-6, TNF-α, IL-1β and PGE2 (39). Fresh CD14+ monocytes in blood showed variable expression of MUC1 that appeared to be donor dependent. However, after the isolation procedure using ficoll gradient and plastic adherence, all monocytes showed upregulation of MUC1 (Fig. 1A). Immature DCs showed expression of HLA-DR, CD80, CD86, CD83, CD40 and CD11c whereby most of these markers were further upregulated upon maturation of DCs. Immature as well as mature DCs were CD14 negative. In culture, DCs showed typical DC morphology with mature DCs having extensive dendrites (Fig. 1B). Immature DCs do not express MUC1, or express at very low levels, depending on the antibody used. The latter is due to the presence of some already mature DCs expressing higher levels of CD86 and HLA-DR in the immature DC population. Moreover, we observed high expression of MUC1 on DCs after full maturation induction. This was demonstrated by two different antibodies specific for MUC1 (Fig. 1B).

We used RT-PCR to show the presence of MUC1 transcripts in DCs. We observed MUC1 transcripts in both the immature and mature DC population (Fig. 2). This confirms MUC1 production by these cells and not just acquisition of shedded

Fig. 1. Expression of MUC1 on fresh and isolated monocytes, and cultured immature and mature DCs. Gates were set to exclude dead cells and debris. Shaded histograms represent the isotype control. Representative samples are shown (n = 10). (A) Fresh monocytes in blood (SSC+, CD14+) show variable levels of MUC1 expression as shown by mAb 214D4. MUC1 is further upregulated after isolation of monocytes (CD14+) using ficoll gradient centrifugation and plastic adherence. Two different donors are shown (S1 and S2). (B) Immature DCs were obtained by culture of monocytes for 6 days in the presence of IL-4 and GM-CSF and further matured by IL-6, TNF-α, IL-1β and PGE2. Immature DCs (dotted line) show expression of HLA-DR, CD80, CD86, CD83, CD40 and CD11c whereby mature DCs (dark line) upregulated further expression of HLA-DR, CD80, CD86, CD83 and CD40. Neither immature nor mature DCs show CD14 expression. The interrupted line shows background staining. In culture, mature DCs show extensive dendrites. Immature DCs (dotted line) did not show MUC1 expression using 214D4 or showed very low levels using DF3, whereas mature DCs (dark line) upregulated MUC1 expression as shown by both anti-MUC1 mAbs, DF3 and 214D4. (C) Immature DCs (dotted line) generated by IL-13 and GM-CSF also showed low levels of MUC1 whereas after induction of maturation by adding Ribomunyl and IFN-γ for another 48 h, mature DCs (dark line) showed further upregulation of MUC1 expression as detected by mAb 214D4.
MUC1. In a separate experiment, monocytes also showed MUC1 transcripts (data not shown).

In order to study if MUC1 expression is a common feature on MoDCs, or dependent on different culture or maturation stimuli, we obtained immature DCs using IL-13 and GM-CSF for 6 days followed by 48 h of maturation using Ribomunyl and INF-γ (33). This resulted in immature and mature DCs expressing the typical DC markers at the same level as in Fig. 1(B) (data not shown). The plot in Fig. 1(C) shows that MUC1 is also expressed on mature DCs prepared according to this method.

Moreover, different maturation stimuli including TNF-α, Poly I:C and a cocktail containing IL-6, TNF-α and IL-1β all resulted in MUC1 upregulation on mature DCs (data not shown).

We analysed MUC1 expression using a variety of antibodies recognizing the fully glycosylated form of MUC1. All these antibodies (DF3, 214D4, HFMG1, HFMG2) demonstrated similar results.

In vivo DCs and MUC1 expression
To investigate whether MUC1 expression on DCs could have a physiological meaning and is not merely an in vitro phenomenon, we analysed in vivo DCs in blood, thymus, tonsil and synovial fluid. First, we checked blood DCs for MUC1 expression. Blood DCs are known to occur in a non-activated state regarding their function to capture antigens followed by maturation and presentation of these antigens to
IL-1 present in an environment with inflammatory cytokines like DCs are known to be partially activated (36,40,41) and visiting the rheumatology clinic were investigated. These negative for MUC1 expression (Fig. 3C).

CD33hi, HLA-DR+, CD11c+ cells) of seven out of twenty patients tested so far turned out to be MUC1 positive (a representative sample is shown in Fig. 3D). In a more detailed analysis of this population, these cells were shown to be also contains a subset of potentially activated DCs (HLA-DRhi, CD11c+), as has been suggested by Summers et al. (38). DC regions were based on high FSC and absence of Lin markers on these cells and subsequently checked for HLA-DR and CD11c expression. All three cell populations, including the potentially activated HLA-DRhi, CD11c+ population, were negative for MUC1 expression (Fig. 3C).

Finally, DCs obtained from synovial fluid from patients visiting the rheumatology clinic were investigated. These DCs are known to be partially activated (36,40,41) and present in an inflammatory environment with cytokines like IL-1β, IL-6 and TNF-α (41,42). Synovial fluid DCs (Lin−, CD33hi, HLA-DRhi, CD11c+ cells) of seven out of twenty patients tested so far turned out to be MUC1 positive (a representative sample is shown in Fig. 3D). In a more detailed analysis of this population, these cells were shown to be also

**Fig. 2.** DCs contain MUC1 mRNA. Immature DCs were obtained by culture of monocytes in the presence of GM-CSF and IL-4 for 6 days and maturation was induced by IL-6, TNF-α, IL-1β and PGE2 (n = 3). RNA was extracted from immature DCs and RNA from the breast cancer cell line ZR75-1 was similarly extracted and subjected to RT-PCR to serve as a positive control. Negative control consisted of MUC1 primers alone without mRNA. Electrophoresis of the RT-PCR products through a 1.2% agarose gel, stained with ethidium bromide resulted in a 351 bp MUC1 specific band.

T cells. In FACS analysis, blood DCs were defined based on Lin−, CD33int and Lin−, CD33hi expression of cells (34,35). These populations include all DCs and did not show MUC1 expression by use of mAb DF3 (n = 2, data not shown).

Three subsets of thymic DCs were defined by FACS analysis to be high FSC, Lin− and HLA-DRhi. This gate contains three subsets: CD11c+ and CD11b+ myeloid DCs and CD123+ plasmacytoid DCs. All three subsets did not show MUC1 expression (Fig. 3A). Since isolation of in vivo DCs might result in loss of these cells, we performed immunohistochemical double staining analysis to minimize manipulation of cells. Indeed, staining of the human thymus confirmed that CD11c+ (red) cells with a morphology suggestive of DCs are not MUC1 (blue) positive (Fig. 3B). However, we observed MUC1 expression on the medullary epithelial cells and the Hassall's corpuscles (our manuscript in preparation).

Tonsils were analysed under the assumption that they contain a subset of potentially activated DCs (HLA-DRhi, CD11c+), as has been suggested by Summers et al. (38). DC regions were based on high FSC and absence of Lin markers on these cells and subsequently checked for HLA-DR and CD11c expression. All three cell populations, including the potentially activated HLA-DRhi, CD11c+ population, were negative for MUC1 expression (Fig. 3C).

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**Discussion**

In this paper we show that in vitro human MoDCs express the tumor-associated antigen MUC1 independent of the culture method or DC maturation cocktail used. Furthermore, we described the expression of MUC1 on in vivo DCs present in synovial fluid. Finally, MUC1 expression can be detected on bone marrow-derived DCs obtained from hMUC1 Tg mice. We confirmed that MUC1 expression on in vitro cultured MoDCs is related to the maturation state of DCs (32). Moreover, MUC1 mRNA can be amplified by RT-PCR confirming MUC1 production, and not acquisition, apparently in both immature and mature DCs. However, because of the presence of already more mature DCs within the immature population, it is likely that the transcribed MUC1 band is derived from DCs expressing already higher levels of CD86 and HLA-DR.

**MUC1 expression in hMUC1 Tg mice corresponds to expression in humans**

hMUC1 Tg mice are frequently used as a model in MUC1 directed immunotherapy. Therefore it is relevant to know if in this model DC show similar expression pattern of MUC1. To determine whether MUC1 is also present on bone marrow-derived DCs in hMUC1 Tg mice, we obtained such DCs by culturing bone marrow cells in the presence of GM-CSF and IL-4 for 7 days, followed by maturation using LPS for another 24 h. FACS analysis using the 214D4 and DF3 mAbs showed MUC1 expression, although at a lower level compared to humans. In concordance with human DCs, mature DCs (CD11c+, HLA-DRhi) of Tg mice express more MUC1 than immature DCs (CD11c+, HLA-DRlo) (Fig. 4). CD68 expression correlated with the HLA-DR expression, providing additional evidence about the maturation stage of the DCs (data not shown).

**No stimulating role for endogenous MUC1 present on the cell surface of MoDCs**

We first established immature DCs by culturing monocytes for 6 days in the presence of IL-4 and GM-CSF and further matured by IL-6, TNF-α, IL-1β and PGE2. The phenotype of these DCs is similar as in Fig. 1(B). When incubation with DF3 was carried out on ice, DCs showed MUC1 expression. In contrast, mature DCs incubated with DF3 at 37°C resulted in downregulation of cell surface MUC1 while DCs incubated with isotype control still expressed MUC1 (Fig. 5A). Moreover, MUC1 expression could not be detected on mature DCs that were incubated for 4 days at 37°C in the continuous presence of anti-MUC1 (Fig. 5B). To determine whether endogenous MUC1 present on the cell surface of MoDCs plays a critical role in the stimulation capacity of autologous, allogeneic or naive T cells, we checked mature DCs expressing MUC1 (isotype incubated at 37°C) or not (DF3 incubated at 37°C) in an MLR. Both type of DCs appeared to induce similar activity (Fig. 6).
Peripheral blood CD14+ monocytes have also been demonstrated to be MUC1 positive (32). We confirmed this observation, and demonstrated that fresh monocytes in blood showed different (low to intermediate) levels of MUC1 expression that are donor dependent, but strongly upregulated after the isolation procedure. This suggests that manipulation of the cells (stress) induces upregulation because MUC1 expression diminishes again upon culture into immature DCs. We confirmed that Lin−, CD33+ DCs present in human blood are MUC1 negative (32) that is in accordance with the concept that MUC1 expression is related to maturation, since blood DCs occur in a non-activated state. In solid organs like the tonsil, we were not able to demonstrate MUC1 expression on DCs, although tonsil DCs appear to have...
a mature phenotype judged by FACS analysis. This indicates that a mature phenotype of DCs in vivo does not necessarily implicate the expression of MUC1. Since there are DCs present in the thymus that are thought to be relevant for tolerance induction, we analysed these cells for MUC1 expression. However, thymic DCs did not express MUC1. On the other hand, we did observe MUC1 expression on medullary epithelial cells (our manuscript in preparation) in accordance with the publication of Gotter et al. who described the presence of various tumor associated antigens on medullary thymic epithelial cells (43). The Hassall’s corpuscles that are composed of tightly packed whorls of epithelial cells also show high expression of MUC1.

We extended the observation that MUC1 is expressed on in vitro derived MoDCs by demonstrating that MUC1 is also expressed on in vivo DCs present in synovial fluid of patients with arthritis. This population of DCs is defined by Lin”, CD33int, HLA-DR”, CD11c” expression and is for the majority CD86”. This is the first observation of in vivo MUC1 expression on DCs, indicating that this is not an in vitro artefact. As shown by Balanescu et al. (40), a highly differentiated subpopulation of DCs present in synovial fluid of patients with clinically active rheumatoid arthritis (Disease Activity Score 28>5.1) express high levels of activation markers like HLA-DR, CD86, CD80, CD83, CD11c, CD54 and CD58. Synovial fluid might resemble the in vitro culture of mature DCs because the ongoing immune response contains the inflammatory cytokines IL-1, IL-6 and TNF-α that leads to further maturation/activation of DCs (41,44) and possible upregulation of MUC1. The Lin”, CD33int population present in synovial fluid does not express MUC1. This
population is regarded to contain mainly precursors for mature DCs. It might be possible that the Lin\(^{-}\), CD33\(^{int}\) DCs will mature and upregulate their MUC1 expression as a response to cytokines in the local inflammation. Therefore one can speculate that MUC1 is not involved in migration of activated DCs into the area of inflammation. In contrast, MUC1 expression on activated T cells has been suggested to be involved in T cell migration. We performed some preliminary experiments on the possible role of MUC1 on DC migration but it is too early to conclude on any functional role for MUC1 (data not shown).

hMUC1 Tg mice are often used for immunotherapeutic strategies, including the design of DC vaccines. Therefore it is relevant to know if MUC1 expression is also present on DCs of these Tg mice. We indeed observed expression of MUC1 on \textit{in vitro} cultured bone marrow-derived DCs. This observation indicates that hMUC1 Tg mice are also in this respect comparable to human regarding their MUC1 expression pattern \textit{in vitro} and that the mouse model is therefore useful for further comparative studies.

It has been speculated that MUC1 on tumor cells down-regulates T cell activity as a result of its extended and rigid structure (45). To determine whether there is a stimulating or inhibiting role for MUC1 present on DCs, we compared mature DCs either or not expressing cell surface MUC1 and analysed their stimulation capacity in an MLR. However, no difference in stimulation capacity of autologous, allogeneic and naïve...
Endogenous membrane-bound MUC1 present on DCs plays no role in DC stimulation capacity to T cells. Immature MoDCs were obtained by culture of monocytes from blood in the presence of GM-CSF and IL-4 for 6 days. Further maturation was induced by incubation with IL-6, TNF-α, IL-10 and PGE2. Mature DCs were preincubated with mAb DF3 (10 μg/ml) or an isotype (10 μg/ml) control for 1 h at 37°C. This resulted in DCs expressing cell surface MUC1 (isotype control incubation) or not (DF3 incubation). Subsequently, resulting DCs were used respectively, in a mixed lymphocyte reaction to test their stimulation capacity to autologous, allogeneic and naive T cells. No difference in T cell proliferation could be determined using thymidine stimulation capacity to autologous, allogeneic and naive T cells. No role in DC stimulation capacity to T cells. Immature MoDCs were obtained by culture of monocytes from blood in the presence of GM-CSF and IL-4 for 6 days. Further maturation was induced by incubation with IL-6, TNF-α, IL-10 and PGE2. Mature DCs were preincubated with mAb DF3 (10 μg/ml) or an isotype (10 μg/ml) control for 1 h at 37°C. This resulted in DCs expressing cell surface MUC1 (isotype control incubation) or not (DF3 incubation). Subsequently, resulting DCs were used respectively, in a mixed lymphocyte reaction to test their stimulation capacity to autologous, allogeneic and naive T cells. No difference in T cell proliferation could be determined using thymidine incorporation. Representative samples of three separate experiments are shown.

T cells could be found. This suggests that MUC1 is not involved in the stimulatory activity that DCs exhibit to T cells. Because MUC1 is expressed by mature/activated DCs in vitro as well as in vivo, this strongly suggests that there is a physiological role for MUC1 on DCs that remains to be further elucidated. ICAM-1 has been described as a ligand for MUC1 (46), and therefore an adhesive function of MUC1 on DCs to ICAM-1 positive cells (T cells or endothelial cells) might exist. It could also be possible that MUC1 is involved in DC-DC interaction via ICAM-1 or other ligands. We are presently studying the binding of MUC1 on DCs to several other ligands. This is the first publication demonstrating MUC1 on in vivo DCs. Identification of the role of MUC1 on human DCs as well as on DC of hMUC1 Tg mice might be useful to understand DC function and for the establishment of an improved protocol for tumor vaccination using (MUC1 and) DCs.

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Abbreviations

AEC 3-amino-9-ethylcarbazole
APC aliphophycocyanin
DC dendritic cell
FCS fetal calf serum
hMUC1 Tg human MUC1 transgenic
IgG1 immunoglobulin G1
LPS lipopolysaccharide
MLR mixed lymphocyte reaction
MoDC monocyte-derived DC
PBMC peripheral blood mononuclear cell
PBS phosphate-buffered saline
PerCP peridinin chlorophyll-a protein
R-PE-Cy5 R-phycocerythrin-Cy5

References

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