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A Novel Tumor-Associated Pancreatic Glycoprotein Is Internalized by Human Dendritic Cells and Induces Their Maturation

Cécile Franceschi,*† Aurélie Collignon,*†,1 Daniel Isnardon,*†,1 Liliane Benkoel,*† Alain Vérine,*† Françoise Silvy,*†,1 Jean-Paul Bernard,*†,6 Dominique Lombardo,*†,6 Evelyne Beraud,*† Daniel Olive,†,6 and Eric Mas*†,6

Aberrant glycosylation or overexpression of cell-surface glycosylated tumor-associated Ags (TAA) distinguish neoplastic from normal cells. Interactions of TAA MUC1 and HER2/neu with dendritic cells (DC) preclude efficient processing, which impairs immune responses. It is thus important to define the mechanisms of interactions between DC and glycosylated TAA and their trafficking and processing for further T cell activation. In this work, we study interactions between DC and the oncofetal fucose-rich glycovariants of bile salt-dependent lipase (BSDL), expressed in pancreatic cancer tissues and referred to as pathological BSDL carrying the fucosylated J28 glycoptope (pBSDL-J28) because it is characterized by the mAb J28. The expression of pBSDL-J28 was assessed by immunohistochemistry and quantified by confocal microscopy. Nontumoral pancreatic tissues and cells do not express pBSDL-J28. Using multidisciplinary approaches and functional studies, we provide the first evidence, to our knowledge, that this tumoral glycoprotein is rapidly internalized by human DC through macropinocytosis and endocytosis via mannose receptors and then transported to late endosomes for processing. Interestingly, pBSDL-J28 per se induced DC maturation with increased expression of costimulatory and CD83 molecules associated with cytokine secretion (IL-8 and IL-6). Surprisingly, DC retained their full ability to internalize Ags, making this maturation atypical. Finally, the allogeneic pBSDL-J28–treated DC stimulated lymphocyte proliferation. Besides, pulsing DC with pBSDL-J28 C-terminal glycopolypeptide and maturation with CD40L triggered CD4+ and CD8+ T cell proliferation. Therefore, interactions of pBSDL-J28, expressed on tumoral pancreatic tissue, with DC may lead to adequate Ag trafficking and processing and result in T cell activation. The Journal of Immunology, 2011, 186: 000–000.

Pancreatic ductal adenocarcinoma (PDAC) is the fourth leading cause of cancer death in Western countries (1). Because conventional therapy is inefficient for PDAC, immunotherapeutic approaches have become alternatives. The development of cancer vaccines is a major challenge for the scientific and medical communities, which have focused on targeted delivery of tumor-associated Ags (TAA) to dendritic cells (DC), the most effective APCs (for reviews, see Refs. 2–4). DC play a central role in immune surveillance. They protect against infection and malignancy by means of TAA processing through the MHC class I and II (MII) pathways and presentation of antigenic peptides to TAA-specific CD4+ and CD8+ T cells (5, 6). For T cell priming and Th response polarization to be efficient, DC must become activated through a maturation process generally characterized by increased expression of maturation markers and costimulatory molecules and by decreased capacities for endocytosis and cytokine secretion (7). DC maturation is thus a critical control point in the initiation of immunity. Cell transformation is often associated with modifications of the glycan moiety of cell membrane glycoproteins (8). Aberrant glycosylation of cell-surface mucins distinguishes neoplastic from normal cells. Glycopeptides are challenging Ags because alteration in the glycan moiety, without any change in the polypeptide backbone, can create neoantigens on cells and affect their interaction with the immune system. CD4+ T hybridoma studies confirmed that O-glycopeptides are presented to T cells and that glycans form integral parts of the TCR-defined epitopes (9). In particular, DC are equipped with specialized receptors, including

1INSERM Unité Mixte de Recherche 911, Centre de Recherche en Oncologie Biologique et Oncopharmacologie, F-13005 Marseille, France; †Campus Sainte-Timone, Aix-Marseille Université, F-13005 Marseille, France; ‡INSERM Unité Mixte de Recherche 891, Centre de Recherche en Cancérologie de Marseille, Infrastructure en Biologie, Santé et Agronomie, Plateforme d’Immunomonitoring, Institut Paoli Calmettes, F-13009 Marseille, France; †INSERM Centre d’Investigation Clinique 95-02, Assistance Publique-Hôpitaux de Marseille, F-13005 Marseille, France; and §Laboratoire d’Immunologie des Tumeurs, Institut Paoli-Calmettes, F-13009 Marseille, France.

A.C. and D.I. contributed equally to this work.

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Address correspondence and reprint requests to Dr. Eric Mas or Dr. Daniel Olive, INSERM Unité Mixte de Recherche 911, Centre de Recherche en Oncologie Biologique et Oncopharmacologie, Faculté de Médecine-Timone, 23 Boulevard Jean Moulin, 13385 Marseille-Cedex 05, France (E.M.) or INSERM Unité Mixte de Recherche 891, Centre de Recherche en Cancérologie de Marseille, 232 Boulevard de Sainte-Marguerite, 13009 Marseille, France (D.O.). E-mail addressess: eric.mas@univmed.fr (E.M.) or daniel.olive@insERM.fr (D.O.).

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Abbreviations used in this article: A488-OVA, Alexa 488-labeled OVA; A488-pBSDL-J28, Alexa 488-labeled pBSDL-J28; BSDL, bile salt-dependent lipase; CLR, carbohydrate ligand receptor; DC, dendritic cell; DC-SIGN, dendritic cell-specific ICAM-3 grabbing nonintegrin; FAPP, fetoacinar pancreatic protein; iMoDC, immature monocyte-derived dendritic cell; LIX-1, lectin-like oxidized low-density lipoprotein-1; MFI, mean fluorescence intensity; MHC, MHC class II; MII, MHC II compartment; MoDC, monocytic-derived dendritic cell; MR, mannose receptor; MSF, mean specific fluorescence; pBSDL-J28, pathological bile salt-dependent lipase carrying the fucosylated J28 glycopeptide; PDAC, pancreatic ductal adenocarcinoma; TAA, tumor-associated Ag.

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Carbohydrate ligand receptors (CLRs), mannos receptor (MR), DC-specific ICAM-3–grabbing nonintegrin (DC-SIGN), and dctin-1, to name but a few, are involved in glycan-mediated pathogen recognition and internalization of endogenous and exogenous Ag for loading on MHC class I and II molecules (15). Targeting Ag to MR leads to enhanced humoral and cellular responses and the induction of tumor-protective immune responses (16). MR has also been demonstrated to play an enhancer role in Ag uptake and presentation, because mannosylated Ags are better internalized and presented to T cells (17–20). However, depending on the DC activation state, Ag presentation to T cells might result in immune activation or immune downregulation (21). For instance, high-mannose structures on the cell wall target MR and DC-SIGN to induce signaling, which leads to the production of anti-inflammatory cytokines such as IL-10 (15). Chieppa et al. (22) reported that DC stimulated through the MR upregulates IL-10 production and downregulates IL-12; later on, they showed that tumor-derived mucins, probably interacting with MR or other C-type lectin receptors on DC, affect the differentiation and maturation of monocyte DC, which result in APCs with a tolerogenic/regulatory cytokine profile (23). These findings exemplify the ability of MR to modulate anti-inflammatory and tolerogenic programs in DC. Yet, the in vivo situation is likely more complex; as shown by others, semimatured DC remains responsive to further signals in vitro and in vivo, which converts their tolerogenicity into immunogeneity (24).

Although the altered tumor-glycosylated Ags represent potential targets for DC-based cancer immunotherapy, the outcome of glycosylated Ag transport, processing, and presentation to T cells is indeed far less explored than that of proteic Ags. In the most studied TAA, mucin MUC1, the aberrantly glycosylated tumor variant in ascites of cancer patients is unprocessed, retained in early endosomes, further released by cells (25), and/or leads to impaired DC differentiation and function (26, 27). Using solely MUC1 peptides, vaccination trials against PDAC led to immune responses and some clinical benefit in phase I study (28–30). Therefore, investigations of the interaction between tumor-specific glycosylated Ag and CLRs on APCs might elucidate why immune responses against tumor glycans are in general so difficult to obtain and might also lead to the development of protocols that generate T cells that are directed against glycan-containing peptides (12). It is thus important to define the mechanisms of interaction between the different TAA glycoforms and the immune cells to develop new immunogens for cancer vaccination.

In this work, we have studied another highly glycosylated Ag, fetoacinar pancreatic protein (FAPP), which is associated to oncodevelopment pancreatic Ag. FAPP is a glycoprotein of bile salt-dependent lipase (BSDL) (31, 32), a lipolytic enzyme present in normal human pancreatic juices (33). FAPP (now referred to as human pathological BSDL carrying the fucosylated J28 glycotope [pBSDL-J28]) was first characterized in human tumoral pancreas (34) and later on in human pancreatic tumor cell lines (35–37) using the murine J28 mAb (mAbJ28). mAbJ28 recognizes a carbohydrate-dependent antigenic structure (31), termed J28 glycopote, located within the O-glycosylated mucin-like C-terminal domain of pBSDL-J28 (38, 39). The formation of the J28 glycopote, characterized by fucosylated O-linked side chains, requires core2 β1,6 N-acetylgalactosaminyltransferase and α1,3/4 fucosyltransferase (39), two glycosyltransferases for which expression is upregulated during pancreatic neoplastic processes (40). This upregulation is in line with the consensual observations that changes occur in posttranslational processes, particularly glycosylation during malignancy (41).

We previously reported the presence of circulating Abs recognizing the O-glycosylated C-terminal domain in most type 1 diabetic patients and some patients afflicted with PDAC (42). This presence may reflect the potential of this O-glycosylated C-terminal domain to induce humoral immunity. The expression of pBSDL-J28 on pancreatic tumoral cells and tissues and their immunogenic potential led us to hypothesize that this glycotope may serve to activate DC and further develop immunotherapy against PDAC. We have therefore investigated the DC processing of tumoral pancreatic glycoprotein pBSDL-J28 and its outcome.

**Materials and Methods**

**Immunohistochemistry**

We used tissue samples from nine cases of primary PDAC (PDAC1–PDAC9). As controls, we used seven nontumoral areas from biopsies of primary malignant and benign pancreas tumors obtained during pancreas resection and two biopsies obtained during transplantation and neurectoderm tumor resection (C1–C9) (Supplemental Table I). Tissue sections were treated as previously described (43).

**Generation of human monocyte-derived DC**

Immature monocyte-derived DC (iMoDC) were generated from PBMC as described (44). PBMC obtained from healthy donors (Etablissement Français du Sang, Marseille, France) were isolated over a Ficoll-Paque gradient (Amersham Pharmacia Biotech, Piscataway, NJ). CD14+ monocytes from PBMC were immunomagnetically purified with CD14 mAb-conjugated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The recovered cells were 99% CD14+ as determined by flow cytometry. Monocytes were cultured at 1 × 10^6 cells/ml in complete RPMI 1640 (Invitrogen, Cergy Pontoise, France) containing GM-CSF (100 ng/ml) and IL-4 (20 ng/ml) (Scherer-Ploug, Kenilworth, NJ) for 5 d to obtain iMoDC. For maturation assays, DC (2–4 × 10^5 cells/ml) were stimulated for 48 h with LPS (5 μg/ml) (Sigma-Aldrich) or pBSDL-J28 (50 μg/ml). The phenotype of DC (CD14+, CD1a+, HLADR+, CD80, CD86, CD38+) was evidenced by flow cytometry.

**Ags**

pBSDL-J28 was purified from human pancreatic juice (32) and directly conjugated to Alexa 488 fluorescent dyes (A488-pBSDL-J28) according to the manufacturer’s protocol (Molecular Probes, Eugene, OR). Alexa 488-labeled OVA (A488-OVA) was used as an uptake control (Molecular Probes). pBSDL-J28 C-terminal glycopeptide was obtained by cyanogen bromide cleavage of pBSDL-J28 (38). The synthetic pBSDL C-terminal polypeptide (77 aa) was purchased from Proteogenix (Oberhausen, Germany).

**Production of rC-terminal polypeptide-J28**

The HEK-C-ter cell line from the HEK 293 cell line (American Type Culture Collection, Rockville, MD) was obtained by transfection with the cDNA encoding the C-terminal domain of FAPP (45), constituted of six tandemly repeated identical sequences (46). These recombinant line cells secreted the C-terminal domain of FAPP carrying the J28 glycopeptide in cell-culture medium. Cells were cultured in Pro293A medium (Lonza, Verviers, Belgium) supplemented with 2 mM glutamine for 16 h. Then, the cell-culture media were withdrawn and concentrated by lyophilization. Dry material was resuspended in water and desalted on a Sephadex G-25 column (10 × 1.5 cm; GE Healthcare). (Glyco)protein fractions were pooled and lyophilized. This material was resuspended in 500 μl PBS, loaded onto a Sephacryl S-200 HR chromatography column (200 × 1 cm; GE Healthcare), and then the material was eluted with PBS. The entire procedure was performed at 4°C. Fractions (1 ml) were collected and analyzed for purity of rC-terminal polypeptide and reactivity to mAbJ28 using SDS-PAGE and Western blot. Reactive fractions containing rC-terminal polypeptide-J28 were pooled, dialyzed against water, and conjugated to Alexa 488 fluorescent dyes (A488-C-ter-J28) according to the manufacturer’s protocol (Molecular Probes).

**Endocytosis assay**

The endocytic activity of iMoDC was measured by the uptake of A488-OVA and A488-pBSDL-J28 at indicated times at 37°C or 4°C. Inhibition assays...
were performed by pretreating iMoDC with cytochalasin D, sacrose, wortmannin, LY29402, amiloride, and mammn (Sigma-Aldrich) or with blocking mAbs to lectin-like oxidized low-density lipoprotein-1 (LOX-1), DC-SIGN, and MR. The treatment was followed by the addition of A488-pBSDL-J28 at 37°C for 1 h. After PBS washes, samples were analyzed by flow cytometry. The mean fluorescence intensity (MFI) of DC was obtained by subtracting the MFI of DC incubated with labeled protein on ice from the MFI of inhibitor-treated and untreated samples. The percent of inhibition was calculated as follows: (MFI of untreated DC + labeled protein) – [MFI of inhibitor-treated DC + labeled protein](MFI of untreated DC + labeled protein) × 100. The uptake of proteins was quantified by flow cytometric analysis using an FACSArray cytometer and FACSArray software (BD Biosciences). Data were analyzed using FlowJo software v-8-3 (Tree Star, Ashland, OR).

Confocal laser scanning microscopy

For quantification of the J28-Ag, tissue sections were treated as described (43). For DC analyses, MoDCs were layered on poly-l-lysine-coated coverslips at a density of 5 × 10⁵ cells/ml in RPMI and pulsed with A488-pBSDL-J28 for indicated times. MoDCs were fixed with 3.7% formaldehyde, permeabilized with 0.1% Triton X-100, counterstained with primary Abs, and revealed with mouse Alexa 594 secondary Ab. Slides were mounted using fluorescent mounting medium. Confocal analyses were performed using a Zeiss LSM 510 microscope (Carl Zeiss). Images were collected sequentially using individual lasers to eliminate bleed through of fluorophores. The percent of colocalization was determined with JACoP as a plugin to the public domain Image J software.

Abs

The surface expression of DC markers was quantified by three-color flow cytometry. In brief, cells were washed and counted prior to incubation with 30% AB human serum (Abcys, Paris, France) for 10 min at 4°C to block nonspecific Fc receptors. Cells were then stained with specific Abs or their isotype-matched controls for 30 min at 4°C in the dark and washed before analysis. Anti-human CD1a-PE and CD83-PE–cyanin5 mAbs (Beckman Coulter, Roissy CDG, France) and CD14-PerCP, HLA-DR–allophycocyanin, DC-SIGN, and MR. The treatment was followed by the addition of A488-pBSDL-J28 for 8–3 (Tree Star, Ashland, OR).

Western blottings

DC were grown in six-well culture plates. Cell pellets were lysed at 4°C in 0.5 ml lysis buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% Triton X-100, 1 mM benzamidine, and phosphatase inhibitor mixture). Homogenates were then cleared by centrifugation (4°C, 10 min, 10,000 × g). An aliquot was saved for protein determination (MicroBCA kit; Pierce, Rockford, IL). Proteins in reducing SDS buffer were separated on 10% polyacrylamide gels and transferred onto a nitrocellulose membrane (Mini Transblot; Bio-Rad, Hercules, CA). The membranes were probed with pAbL64 or mAbJ28. Detection and development were performed as previously described (39). In each experiment, a control was included by omitting primary Abs.

Cytokine measurements by cytometric bead array

DC supernatants were collected at day 7 of culture, and cytokine secretion ([IL]-1β, IL-6, IL-8, IL-10, IL-12, and TNF-α) was determined using the Human Inflammation CBA Kit (BD Biosciences). Supernatants of lymphocyte/DC allogeneic coculture were collected at days 2 and 6. Cytokine secretion ([IL]-4, [IL]-5, [IL]-10, [IL]-17, [IL]-21, and IFN-γ) was determined by flow cytometry using Th1/Th2 Flex Sets 6 plex and quantified using FCAP array software (BD Biosciences).

MLR

CD4⁺ T cells were generated from PBMC by negative sorting using naive CD4⁺ T cell isolation kit (Miltenyi Biotec). DC were untreated or previously stimulated with LPS (5 μg/ml) or pBSDL-J28 (50 μg/ml) for 48 h. CD4⁺ T cells (2 × 10⁵ cells/well, responder cells) were incubated for 2 and 6 d with DC (stimulator cells) at a D/C/T cell ratio of 1:10 in 96-well round-bottom plates.

CFSE labeling for T cell proliferation assays

CD4⁺CD45RA⁺ T cells were labeled with 0.5 μM CFSE (Vybrant CFDA-SE Cell Tracer Kit; Molecular Probes) (10 min, 37°C) and washed three times in cold PBS and complete medium before being placed in culture. MLR was performed as described above for 5 d. T cell proliferation was quantified by flow cytometry. FlowJo’s proliferation platform (Tree Star) was used to analyze T cell proliferation, where the division index is the average number of CD4⁺ T cell divisions that the responding cells underwent.

FIGURE 1. Expression of J28-glycosylated Ags on tumoral human pancreatic tissues. A. Immunohistochemical staining with mAbJ28 of case 2 (PDAC2) and case 5 (PDAC5) of pancreatic tumors and from case 2 (C2) and case 4 (C4) of nontumoral pancreas. Areas marked within squares are representative of each case were measured. Medians of the MSF intensities obtained with mAbJ28 were compared in each case. Differences were considered significant at p < 0.05. **p < 0.001.

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MoDC were preincubated with pBSDL-J28 C-terminal glycopolypeptide or synthetic pBSDL C-terminal polypeptide (50 \( \mu \)g/ml) overnight, and then exposed to CD40L (48) for 1 d in medium containing 10% serum AB, IL-4, and GM-CSF in 24-well plates at 0.5 \( \times 10^6 \) cells/well. CD3\(^+\) T cells were generated from PBMC by negative sorting using the Pan T-Cell Isolation Kit II (Miltenyi Biotec). Naive CD3\(^+\) T cells were cultured with mature MoDCs at a ratio of 10:1 and plated in 24-well plates at 2 \( \times 10^5 \) T cells/well. T cells were restimulated weekly with Ag-pulsed autochthonous MoDC and maintained in 30 U/ml IL-2 and 10 ng/ml IL-7; On the third stimulation, T cells were seeded in 96-well plates at 1.5 \( \times 10^5 \) with MoDC at a ratio of 1:1, cultured for 7 d in the same conditions as above, and labeled with CFSE for cell proliferation detection. Before fluorescence analysis, CD3\(^+\) T cells were stained with CD4-PC7 and CD8-allophycocyanin Abs (Beckman Coulter).

### Results

#### Quantification of J28-glycosylated Ags on tumoral pancreatic human tissues

To determine the expression of pBSDL-J28 in pancreatic tissues, tumoral and nontumoral tissues were incubated with mAbJ28. Labeling of membranes and around the neoplasm was observed for PDAC2 (Fig. 1A, PDAC2 enlargement). For PDAC5, the luminal membrane of cells forming ducts was highly fluorescent, whereas labeling was diffuse in the external part of the tumoral tissue (Fig. 1A, PDAC5 enlargement). In control cases (C2 and C4), slight staining was localized in a few areas (Fig. 1A). To quantify the expression of pBSDL-J28, confocal laser scanning microscopy analyses were performed on nine PDAC and nine...
controls. Box plots show that the average mean specific fluorescence (MSF) intensity of mAbJ28 staining for PDAC (median 78.64) was significantly higher than for controls (median 8.00) (Fig. 1B).

**Uptake of pBSDL-J28 by MoDC**

The presence of pBSDL-J28 on tumoral tissues led us to hypothesize that this glycoprotein may serve as tumoral Ag for raising immunological responses using DC. To investigate whether pBSDL-J28 could be internalized, iMoDC were incubated with A488-pBSDL-J28 at various concentrations and times at 37°C. Both A488-pBSDL-J28 and A488-OVA (uptake control) were captured (Fig. 2A). The uptake of A488-pBSDL-J28 was time and concentration dependent. To confirm the efficiency of the uptake, iMoDC incubated with A488-pBSDL-J28 or A488-OVA were analyzed by confocal microscopy. Fluorescence within iMoDC was intense, corresponding to the presence of A488-pBSDL-J28 and A488-OVA (green) (Fig. 2B). These results were reproduced using the rC-terminal polypeptide-J28 labeled with A488, indicating that: 1) interactions between the glycosylated moiety and iMoDC were sufficient to trigger Ag capture; 2) A488 binding to the glycosylated moiety did not impair its capture; and 3) putative ligands carried by the N-terminal portion of glycoprotein pBSDL-J28 were not necessary for DC receptors (Supplemental Fig 1).

In contrast, no colocalization of CD1a (an iMoDC membrane marker) was observed with either pBSDL-J28 or OVA. To determine how A488-pBSDL-J28 is internalized, inhibition experiments were done using inhibitors of receptor-mediated endocytosis and macroinocytosis, two main pathways by which DC internalize Ags for consecutive presentation to naive T cells. To inhibit active endocytosis, we used cytochalasin D, which disorganizes the actin network, and sucrose, which interferes with clathrin-coated pit formation. Preincubation of iMoDC with these inhibitors partially inhibited the uptake of A488-pBSDL-J28 (50 and 19%, respectively) (Fig. 2C). Treatment of iMoDC with LY294002 and wortmannin, two inhibitors of phosphoinositide 3-kinase (PI3K involved in signaling of macropinocytosis), led to marked uptake inhibition, reaching 3-15% and 3-15% inhibition, respectively (Fig. 2C). These inhibition experiments suggest that iMoDC internalize pBSDL-J28 by macropinocytosis, but receptor-mediated endocytosis cannot be excluded. We next attempted to identify putative receptor(s) involved in the uptake of pBSDL-J28. We used specific mAbs blocking MR, DC-SIGN, LOX-1 [a scavenger receptor implicated in cross-presentation of exogenous Ags and enterocytic uptake of BSDL (49)], and mannann, an MR and DC-SIGN competitor. Pretreatment of iMoDC with blocking Abs to LOX-1 and DC-SIGN did not markedly inhibit A488-pBSDL-J28 uptake (19 and 8% inhibition, respectively) (Fig. 2C). The uptake of pBSDL-J28 was significantly inhibited after pretreatment with mannann and MR-blocking Abs (83 and 89% inhibition, respectively). Overall, these results indicated that pBSDL-J28 was internalized by MoDC through two main mechanisms, macroinocytosis and MR-mediated endocytosis.

**Trafficking and degradation of pBSDL-J28 in MoDC**

Exogenous peptides are generally processed by DC and loaded onto MII. To study the intracellular localization of A488-pBSDL-J28 in iMoDC, various markers of lysosomes and early and late endosomes were used. After 10 and 30 min of DC challenging with A488-pBSDL-J28, a small fraction of A488-pBSDL-J28 colocalized with CD206 at the cell surface and in early endosomes (Fig. 3A). This colocalization disappeared after 1 h uptake. At that time, A488-pBSDL-J28 showed intense fluorescence in perinuclear vesicles, whereas the MR was still located at the plasma membrane vicinity. These data revealed that the fraction of MR-interacting pBSDL-J28 was dissociated from receptors within 1 h after pBSDL-J28 uptake. HLA-DR staining was intense in perinuclear vesicles, corresponding to late endosomes and MII compartment (MIIC), whereas fluorescence was faint at the
plasma membrane (Fig. 3B). The three-color overlay analysis indicated a theoretical colocalization (90%) of A488-pBSDL-J28 and HLA-DR. Furthermore, we observed that A488-pBSDL-J28 colocalized with HLA-DM (80% of overlay) and with CD107a in lysosomes (50% of overlay) (Fig. 3B). All of these data support the notion that, after its uptake, pBSDL-J28 was quickly transported to MIIC and lysosomes. Afterwards, we investigated whether pBSDL-J28 was degraded for processing. DC lysates were prepared after pBSDL-J28 incubation. After 1 h incubation, DC lysates contained a 110-kDa protein recognized by both mAbJ28 and pAbL64 (which recognize both BSDL and pBSDL-J28) corresponding to the native pBSDL-J28 (Fig. 3C). After 48 h incubation, pBSDL-J28 reactivity disappeared, suggesting that this protein was totally degraded.

Expression of maturation markers by pBSDL-J28–treated MoDC

As expected, decreased CD14 expression was observed after differentiation of monocytes into DC (Fig. 4A). During pBSDL-J28 uptake by iMoDC, cell-surface translocation of MII molecules increased in 40% of pBSDL-J28–treated MoDC (Fig. 4B). This finding suggested that pBSDL-J28 could induce MoDC maturation per se. Thus, we investigated the effects of pBSDL-J28 on phenotypic and functional maturation of MoDC. iMoDC were stimulated for 48 h with pBSDL-J28 or LPS, an inducer of maturation (control). Stimulation with pBSDL-J28 resulted in increased expression of CD80, CD86, CD83, and MII molecules compared with untreated iMoDC (Fig. 4C). This increase in co-stimulation and maturation markers was close to that induced by LPS, in particular MII molecules, which was even superior in a few cases. To verify that the pBSDL-J28 solution was not contaminated by LPS or endotoxins, we tested on the MoDC phenotype the effects of elution buffer and of different protein fractions obtained during the pBSDL-J28 purification processes. No expression of CD80, CD83, CD86, and HLA-DR was observed (data not shown). Moreover, we examined whether the interaction between pBSDL-J28 and MR was required to induce the expression of these maturation markers. The cell-surface expression of maturation markers (Fig. 4C) was decreased in the
presence of MR-blocking Ab, indicating that the induction of DC maturation depended on the binding of pBSDL-J28 to MR.

**Functional characteristics of pBSDL-J28–treated MoDC**

**Cytokine secretion by pBSDL-J28–treated MoDC.** Because MoDC maturation is associated with cytokine secretion, the culture medium of MoDC was analyzed for the presence of proinflammatory cytokines. Interestingly, IL-8 and IL-6 levels were significantly higher when MoDC were treated with pBSDL-J28 instead of medium (despite the fact that, for the donors 1 and 6, pBSDL-J28 induced neither augmentation of marker expression nor marked cytokine secretion from MoDC, contrary to positive control LPS-treated MoDCs) (Fig. 5A). Moreover, the production of IL-8 significantly correlated with CD86 expression when MoDC from six donors were treated with pBSDL-J28 (Pearson’s coefficient = 0.886; \( p = 0.019 \)); no significant correlation was found for any other combinations of marker expression and cytokine secretion. No substantial production of IL-12, IL-10, and TNF-\( \alpha \) was observed. When MoDC were treated with LPS, they secreted high levels of IL-8, IL-6, and TNF-\( \alpha \) (Fig. 5A) and variable levels of IL-12 (10–9000 pg/ml) associated to low levels of IL-10 and IL-1\( \beta \) for each donor (data not shown). No significant correlation could be established between marker expression and cytokine secretion. Lastly, the comparison of the IL-8 medians of pBSDL-J28–treated MoDC (45303, range from 7483–63240 pg/ml) and LPS-treated MoDC (28333, range from 17448–60650 pg/ml) from different donors indicated a marked tendency for pBSDL-J28–treated MoDC to secrete more IL-8 than LPS-treated MoDC. In contrast, the profile of cytokine secreted by LPS-treated MoDC is dominated by TNF-\( \alpha \).

**Ag uptake by pBSDL-J28–treated MoDC.** We investigated whether pBSDL-J28–treated MoDC had reduced endocytic activity, a functional characteristic of mature MoDC. iMoDC were exposed (48 h) with pBSDL-J28 and then incubated (1 h) with OVA-A488. Surprisingly, uptake at 37˚C of OVA-A488 by pBSDL-J28–treated MoDC was similar to that of untreated iMoDC (Fig. 5B). As

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**FIGURE 5.** Functional characteristics of pBSDL-J28–treated MoDC. A, Cytokine secretion by pBSDL-J28–treated MoDC. Levels of cytokine production of untreated MoDC (solid columns), LPS-treated MoDC (open columns), and pBSDL-J28–treated MoDC (hatched columns) from donor 1 to donor 6 were quantified in 48-h culture supernatant by flow cytometry. Comparisons were made for IL-8 and IL-6 secretion by pBSDL-J28–treated MoDC versus untreated MoDC or LPS-treated MoDC versus untreated MoDC by Wilcoxon test. Differences between groups were considered significant at \( p < 0.05 \). B, Ag uptake by pBSDL-J28–treated MoDC. iMoDC were incubated for 48 h with pBSDL-J28 or LPS. OVA-A488 uptake was measured by flow cytometry; purple line is for A488-pBSDL-J28–treated MoDC, and blue line is for LPS-treated MoDC. Untreated cells were incubated at 4˚C (green line) or 37˚C (orange line). The shaded areas represent the histogram of the nonfluorescent cells. The results are representative of three independent experiments.
expected, the uptake of OVA-A488 by LPS-treated MoDC was markedly decreased. Thus, endocytosis was unaffected by MoDC pretreatment with pBSDL-J28, although these MoDC harbored the hallmark of mature DC. Hence, pBSDL-J28 was able to induce an atypical maturation of MoDC.

*pBSDL-J28 favors the generation of mature DC with increased T cell stimulatory capacity.* The mature cell-surface phenotype and the increased IL-8 production induced by pBSDL treatment prompted us to investigate the T cell allostimulatory capacity of pBSDL-J28–treated DC. When naive CFSE-labeled CD4* T cells were incubated with allogeneic pBSDL-J28–treated MoDC, CFSE fluorescence was decreased, correlating with proliferation of CD4* T cells. In contrast, naive CFSE-labeled CD4* T cells incubated with allogeneic untreated MoDC underwent slow growth (Fig. 6). Box plots show that the division index of naive T cells was significantly greater with pBSDL-J28–treated MoDC than that obtained with untreated MoDC. Next, production of various cytokines (IL-4, IL-5, IL-10, IL-17, and IFN-γ) was determined in the supernatants of three combinations of donors. One of them exhibited a substantially higher IFN-γ level than untreated MoDC (4499 pg/ml versus 2306 pg/ml); no other cytokines were detected (data not shown). Hence, pBSDL-J28 triggered atypical DC maturation but still might allow T cell activation.

**CD4* and CD8* T cell activation triggered by pBSDL-J28 C-terminal glycopolypeptide-treated CD40L-matured DC.** To favor optimal DC maturation, MoDC were exposed to the potent maturation agent CD40L. The analysis by flow cytometry of cell-surface phenotype shows high expressions of HLA-DR, CD80, CD86, and CD83 in comparison with those of immature MoDC, as expected (Supplemental Fig. 2A). After pulsing with pBSDL-J28 C-terminal glycopolypeptide, mature MoDC were able to induce CD4* and CD8* T cell proliferation, whereas MoDC pulsed with synthetic pBSDL–C-terminal polypeptide did not (or hardly with regard to CD8*CD3* T lymphocytes) compared with control mature MoDC without pulsing (Supplemental Fig. 2B, 2C).

**Discussion**

Whereas the expression of pathological pBSDL-J28 in pancreatic cancers represents potential targets for DC-based cancer immunotherapy, the outcome of glycosylated Ag transport and processing and the ability to DC induce maturation require peculiar investigations. Our results show that iMoDC captures pBSDL-J28 as well as C-terminal polypeptide-J28. DC internalize tumoral Ags mainly by macropinocytosis and receptor-mediated endocytosis to direct them to MIIC (50). Signaling pathways, including that of macropinocytosis, are blocked by PI3K inhibitors or by an antagonist of the Na+/H+ pump (51). Thus, our results point out the role of macropinocytosis in the uptake of pBSDL-J28 by iMoDC. Moreover, because macropinocytosis was inhibited to some extent with cytochalasin D, we thought that membrane receptor(s) could be involved too. We found that pBSDL-J28 binds to MR expressed on the iMoDC surface. This binding could be inhibited by Abs to MR or by mannan. Thus, oligosaccharides recognized by C-type lectin-like carbohydrate recognition domains of MR terminating in mannos, N-acetylglucosamine, and/or fucose residues, the latter being crucial elements of the J28 glycotope structure for mAbJ28 recognition, can be involved in DC binding (52). However, we cannot rule out that pBSDL-J28 binds to other receptors. This binding results in the internalization of pBSDL-J28 and its delivery into MIIC and late endosomes, where the A488-pBSDL-J28–processing products were detected. A488-(glycosylated)-peptide epitopes colocalized in lysosomes with LAMP-1 and in late endosomes with HLA-DM. The latter molecular chaperone is known to influence the selection of peptides that bind to MIIC by favoring the presentation of high-stability peptides (53, 54).

We provide evidence that pBSDL-J28 can be delivered into the HLA class II pathway, as opposed to MUC1 and HER2/Neu (25), which present differently glycosylated structures. In PDAC, pBSDL-J28 carries fucosylated O-linked branches, whereas tumoral
MUC1 carries short O-glycans such as T (Galβ1-3GalNAcα1-O-Ser/Thr), Tn (GalNAcα1-O-Ser/Thr), and sialyl-Tn Ags (NeuAc-GalNAcα1-O-Ser/Thr) (8, 55). Several apparently contradictory reports have illustrated the complexity of glycoantigen binding and internalization. MUC1 binding and internalization involve the presence of sialyl (55) and GalNAc residue at terminal position (8), which require Siglecs and macrophage galactose-type lectin receptors, respectively, and can drastically change the binding affinity of ligand to receptor. These data confirm that the TAA glycosylation pattern plays an important role in Ag internalization, processing, glycosylated epitope presentation, and subsequent immune response. Thus, in addition to regulating cell-cell adhesion and migration, the alteration of carbohydrates on the cell surface during carcinogenesis might influence the interaction with CLRs on resident APCs and direct the response toward T lymphocyte activation or inhibition (12).

pBSDL-J28 induced the expression of MII molecules, costimulatory molecules CD80 and CD86, and maturation marker CD83. This agrees with data showing heightened expression of these markers by DC once activated by recombinant sialylated MUC1 (55). The phenotypic maturation of MoDC activated by pBSDL-J28 was associated with the secretion of proinflammatory cytokines IL-8 and IL-6 (but not IL-12p70, IL-10, and IL-1β). IL-8 and IL-6 are known to recruit neutrophils, macrophages, and NK cells, which are responsible for tumor cell clearance (56–58). Yet, these two cytokines contribute to multiple protumorigenic activities (59). Actually, dual actions of IL-8 are well illustrated during tumor progression (59). IL-6, together with TGF-β, is also required for Th17 cell development, which is responsible for adaptive immunity responses. However, IL-17 generally favors the growth of tumors, and this effect outweighs its role in the generation of T cell antitumor immunity (60).

It is reported that IL-8, IL-6, and IL-10 are significantly elevated in sera of patients with PDAC compared with controls (61, 62). Intriguingly, human pancreatic cancer cell lines produce IL-8 and IL-6 (61). Pasare and Medzhitov (63) demonstrated that DC function is impaired in pancreatic cancer patients. They found that one of the soluble factors released by pancreatic cancer cells and being responsible for inhibiting DC differentiation and activation is IL-8, more recently, Tjomsland et al. (64) evidenced the implication of the inflammatory factor PGE2 in patients with ductal pancreatic adenocarcinoma, causing semi mature blood DC. Thus, the immunosuppressive microenvironment created by tumors themselves through the release of soluble factors renders DC tolerant (63–65). At present, we do not know whether IL-8- and IL-6-secretting (but not IL-10) pBSDL-J28 mature MoDC could be generated in vivo and take part in the suppressor environment within tumoral and inflammatory pancreas.

In terms of MoDC cytokine production, pBSDL-J28 activation was mainly characterized by IL-8 secretion, whereas LPS activation was associated with TNF-α, IL-6, and IL-12 secretions. However, in terms of upregulation of CD86, CD80, and CD83 by MoDC, LPS challenge was more efficient than that of pBSDL-J28. Lastly, IL-8 secretion showed the only significant correlation with CD86 expression, making pBSDL-J28 activation different. As a whole, there is no compelling evidence for a cytokine context directing T cells to Th1 polarization.

Maturation of MoDC by pBSDL-J28 was associated with increased expression of MII molecules and CD83, which are stimulators of CD4+ T cell proliferation in allogeneic MLR. T cell proliferation, although mild, was induced with MoDC treated by pBSDL-J28. The cytokine profile of CD4+ T cells in MLR cultures in the presence of pBSDL-J28–activated MoDC points out the production of IFN-γ, although not for each donor. IFN-γ production is also the hallmark of MLR with allogeneic DC treated with HEK MUC1 (55). Lastly, the lack of IL-4, IL-5, IL-10, and IL-17 indicates a lack of Th2, Th17, and Treg induction. These observations suggest that Th1-like cytokine production is favored under allostimulation.

A closer examination shows that MoDC treated with pBSDL-J28 followed an atypical, incomplete maturation process. Although one characteristic of DC maturation is a reduced ability for Ag uptake, MoDC after pBSDL-J28 internalization were surprisingly still able to take up OVA. To our knowledge, there is no report to date on the ability of mature MoDC to internalize Ag. Overall, however, our findings provide evidence for MoDC maturation upon pBSDL-J28 challenge. Licensing of DC by CD40 ligation remains the way to induce IL-12 production and subsequent Th1-polarized response (66), which is one of the objectives for cancer vaccination. To obtain full MoDC maturation and better subsequent T cell activation, a combination of CD40 binding and pBSDL-J28 should therefore be applied, which might more clearly skew the immune response toward Th1 polarization. This proved promising as pulsing DC with pBSDL-J28 C-terminal glycopolyptide and maturation with CD40L-triggered CD4 and CD8 T cell proliferation, highlighting also the existence of TCR(s) for pBSDL-J28 O-glycosylated C-terminal domain in the T cell repertoire.

The demonstration that pBSDL-J28 is efficiently bound to MR, is also internalized by macrophinocytosis, and further delivered to the MHC makes it a potential mechanism for the targeting of DC and the delivery of tumor-associated epitopes for presentation via MII molecules. Thus, this study yields novel and unexpected information regarding the fate of a glycoprotein marker for pancreatic cancer after binding to DC.

Understanding the mechanisms of interaction between TAA and DC paves the way for further strategies to induce appropriate Th1 immune responses against pBSDL-J28–bearing tumor cells in view of therapeutic advantages.

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