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Involvement of LOX-1 in Dendritic Cell-Mediated Antigen Cross-Presentation

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Summary

Some exogenous antigens, such as heat shock proteins or apoptotic bodies, gain access to the MHC class I processing pathway and initiate CTL responses, a process called cross-priming. To be efficient in vivo, this process requires endocytosis of the antigen by dendritic cells via receptors which remain unidentified. Here, we report that scavenger receptors are the main HSP binding structures on human dendritic cells and identify LOX-1 as one of these molecules. A neutralizing anti-LOX-1 mAb inhibits Hsp70 binding to dendritic cells and Hsp70-induced antigen cross-presentation. In vivo, to target LOX-1 with a tumor antigen using an anti-LOX-1 mAb induces antitumor immunity. Thus, the scavenger receptor LOX-1 is certainly a promising target for cancer immunotherapy.

Introduction

T cell epitopes derived from exogenous antigens and endocytosed by antigen-presenting cells (APC) are mainly loaded into the MHC class II molecules for recognition by $CD4^+$ T cells. In some conditions, APC are able to present exogenous antigen in the context of the MHC class I molecules to $CD8^+$ T cells, a process called cross-presentation (Yewdell and Bennink, 1999; Heath and Carbone, 2001). It is thought that antigens have to be taken up via endocytic receptors to be efficiently cross-presented in vivo (Arnold-Schild et al., 1999; Wassenberg et al., 1999; Singh-Jasuja et al., 2000; Castellino et al., 2000). Among the APC, dendritic cells (DC) are the only ones able to prime naive T cells and to generate cytotoxic responses (Banchereau et al., 2000). Thus, to identify receptors expressed by DC and involved in Ag cross-priming is currently a challenge in antitumor immunotherapy.

Heat shock proteins (HSP) are molecular chaperones that control the folding and prevent the aggregation of proteins (Feldman and Frydman, 2000). Previous studies have reported that tumor-derived HSP initiate protective and tumor-specific CTL responses (Tamura et al., 1997). The cellular and molecular mechanisms involved have been partially identified: HSP noncovalently complex tumor Ag-derived peptides (Udono and Srivastava, 1993; Suto and Srivastava, 1995; Blachere et al., 1997) and bind to DC and macrophages (Arnold-Schild et al., 1999; Wassenberg et al., 1999; Todryk et al., 1999) before being internalized in a receptor-dependent manner (Arnold-Schild et al., 1999; Wassenberg et al., 1999; Singh-Jasuja et al., 2000; Sondermann et al., 2000). HSP then colocalize with MHC class I molecules in the early and late multivesicular endosomal structures (Arnold-Schild et al., 1999; Wassenberg et al., 1999; Singh-Jasuja et al., 2000).

Several studies have focused on the identification of HSP binding structures expressed at the surface of APC. Recently, CD91 (also known as the α2 macroglobulin [α2M] receptor) has been reported to be a receptor for human Hsp70, Hsp90, and the endoplasmic reticulum chaperone gp96 on macrophages (Binder et al., 2000; Basu et al., 2001). CD91 is a member of the lipoprotein receptor family expressed on a variety of cells including macrophages and smooth muscle cells (Herz and Strickland, 2001). CD91 is also involved in gp96-mediated peptide re-presentation by macrophages (Binder et al., 2000). Although highly expressed on macrophages, CD91 expression is very low on dendritic cells. We then used Hsp70 as a probe to identify receptors expressed on dendritic cells and involved in HSP-mediated antigen cross-presentation. We show here that scavenger receptors, and especially LOX-1, are the main HSP binding structures on human dendritic cells and that LOX-1 is involved in in vivo antigen cross-priming.

Results

Existence of HSP Binding Elements on Human Dendritic Cells

FACS analysis showed that biotinylated Hsp70 bound to peripheral blood myeloid DC in a dose-dependent manner, significant at 1 nM (MFI = 8 ± 3; mean ± sd, n = 5) and maximal at the highest concentration tested, 500 nM (MFI = 95 ± 12) (Figure 1A). No binding of biotinylated tetanus toxoid (TT), used as a negative control, was detected (Figure 1A). Similar results were obtained using $Alexa^{488}$-labeled Hsp70 and TT (MFI = 85 ± 7 and 3 ± 2, using 200 nM $Alexa^{488}$-labeled Hsp70 and TT, respectively; mean ± sd, n = 5). Hsp70 binding to DC was saturable (Figure 1A) and partly competed by unlabeled Hsp70 (Table 1) but not by TT or BSA either unlabeled (Figure 1A and Table 1, respectively) or biotinylated (data not shown). Hsc70 bound in a similar manner to Hsp70 to peripheral blood myeloid DC (MFI =

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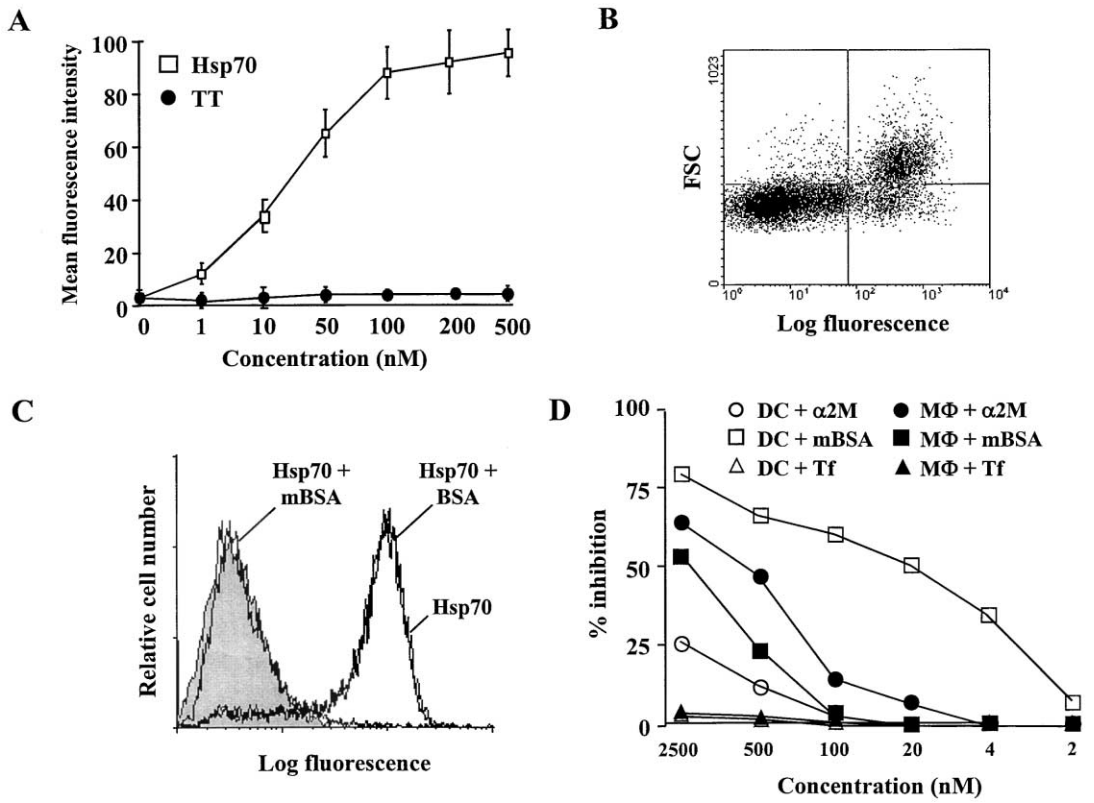

Figure 1. Scavenger Receptors Are Binding Structures for Hsp70

(A) Hsp70 binds to human DC by a mechanism with the characteristics of a saturable receptor system. Purified human peripheral blood immature DC were incubated for 20 min with 1 to 500 nM biotinylated Hsp70 (□) or TT (●) revealed with Alexa^{488}-labeled streptavidin. Fluorescence was analyzed by FACS. Results are expressed in MFI values as mean ± sd, n = 5.

(B) Dot plot profile showing the binding of Hsp70 to human PBMC. Purified human PBMC were incubated for 20 min with 200 nM biotinylated Hsp70 revealed by Alexa^{488}-labeled streptavidin. Fluorescence was analyzed by FACS.

(C) Maleylated BSA inhibits Hsp70 binding to immature DC. Human immature DC were incubated with 2 μM mBSA or BSA. After 20 min, 20 nM biotinylated Hsp70 was added and revealed with Alexa^{488}-labeled streptavidin before analysis by FACS. Results are representative of one out of five experiments. Gray histrogram corresponds to cells incubated with Alexa^{488}-labeled streptavidin.

(D) Inhibition of Hsp70 binding to human macrophages (Mϕ) and peripheral blood myeloid DC. Peripheral blood myeloid DC (open labels) and macrophages (filled labels) were incubated with 4–2500 nM of α2M (circle), mBSA (square), or transferrin (triangle) (used as control proteins) before incubation with 20 nM biotinylated Hsp70 revealed with Alexa^{488}-labeled streptavidin. Results are expressed in percentage of inhibition. One representative from five experiments is presented.

Table 1. Inhibition of Biotinylated Hsp70 Binding to Human Macrophages and Immature Dendritic Cells

Molecules	Concentration	% Inhibition of Hsp 70 Binding		
		Immature DC	Macrophages	LOX-1-CHO
Hsp70	4 μM	55 ± 10	59 ± 11	85 ± 7
	0.1 μM	17 ± 9	9 ± 8	nd
Maleylated BSA	2.5 μM	80 ± 10	55 ± 9	96 ± 5
	0.1 μM	61 ± 8	11 ± 6	nd
BSA	2.5 μM	4 ± 3	3 ± 2	5 ± 4
Ox-LDL	2.5 μM	75 ± 8	45 ± 8	66 ± 8
Ac-LDL	2.5 μM	67 ± 10	52 ± 9	52 ± 7
ApoB	2.5 μM	45 ± 8	47 ± 8	70 ± 5
Poly [I]	100 μg/ml	94 ± 6	91 ± 5	90 ± 5
Poly [A]	100 μg/ml	4 ± 2	4 ± 3	6 ± 4
Fucoidan	50 μg/ml	86 ± 9	78 ± 7	85 ± 10
α-CD14 mAb	50 μg/ml	5 ± 2	4 ± 5	6 ± 3
Control mAb	50 μg/ml	4 ± 3	5 ± 6	5 ± 4
α2M	2.5 μM	27 ± 5	65 ± 6	nd
	0.1 μM	2 ± 3	18 ± 8	nd

Following incubation with the indicated molecules, cells were washed and further incubated with 20 nM biotinylated Hsp70 followed by Alexa^{488}-labeled streptavidin. Binding of Hsp70 was analyzed by FACS. Results are representative of five separate experiments and expressed in % of inhibition of binding (mean ± sd). nd, not done.

Table 2. Hsp70 Binds to Human and Mouse APCs

Origin	Cell Type	Hsp70 Binding (MFI Values)
Human	Monocytes	89 ± 10
	Macrophages	125 ± 19
	Myeloid DC	90 ± 12
	Mature DC	20 ± 7
	B lymphocytes	15 ± 4
	T lymphocytes	3 ± 4
Mouse	DC	65 ± 9

Binding of biotinylated Hsp70 to different human cell types and to mouse immature DC was analyzed by FACS. Cells were successively incubated with 200 nM biotinylated Hsp70 and Alexa^{488}-labeled streptavidin and then analyzed by FACS. Results are expressed in MFI values (mean ± sd, n = 5). Human DC are CD11c^{+} peripheral blood myeloid DC (freshly purified or treated for 48 hr with the maturation factor LPS).

88 ± 10 using 200 nM biotinylated Hsc70; mean ± sd, n = 5) (data not shown). These data suggest that Hsp70 binding structures exist on human APCs (Arnold-Schild et al., 1999; Wassenberg et al., 1999; Singh-Jasuja et al., 2000; Castellino et al., 2000, Sondermann et al., 2000).

As previously reported (Arnold-Schild et al., 1999), biotinylated Hsp70 also bound to subpopulations of peripheral blood mononuclear cells (identified by double-color FACS analysis as CD14^{+} monocytes and CD19^{+} B cells) (Figure 1B), to purified human monocytes, macrophages (Arnold-Schild et al., 1999; Wassenberg et al., 1999; Singh-Jasuja et al., 2000; Sondermann et al., 2000), and, to a lower extent, to B cells and mature DC but not to T cells (Table 2 and Castellino et al., 2000). Interestingly, Hsp60 and Hsp90 presented a similar pattern of binding (data not shown).

Although the Hsp70 used contained low levels of endotoxin (<0.5 EU/mg as assessed by the limulus amebocyte lysate assay), we excluded a potential involvement of residual endotoxin in Hsp70 binding to APC based on the following observations: (1) heat treatment of Hsp70 (100°C for 20 min) totally abolishes its binding to APC and (2) polymixin B and LPS do not prevent Hsp70 binding (data not shown). The involvement of CD14 was suspected in HSP-mediated APC activation (Asea et al., 2000). A neutralizing anti-CD14 mAb (clone MY4) did not modulate Hsp70 binding to DC or macrophages (Table 1), and Hsp70 did not bind to CD14-transfected cells (Figure 2). These data do not support a role for CD14 as an Hsp70 receptor. Previous studies reported that CD91 is a ligand for Hsp70, Hsp90, gp96, and calreticulin on macrophages (Binder et al., 2000; Basu et al., 2001). Although Hsp70 bound to human macrophages and peripheral blood myeloid DC (Table 2), CD91 was detected by FACS on macrophages but not on DC (MFI = 37 ± 5 and 5 ± 4, respectively; mean ± sd, n = 5) (data not shown). Together, these data show that HSP binding elements exist on human immature DC and suggest that they could differ from CD91.

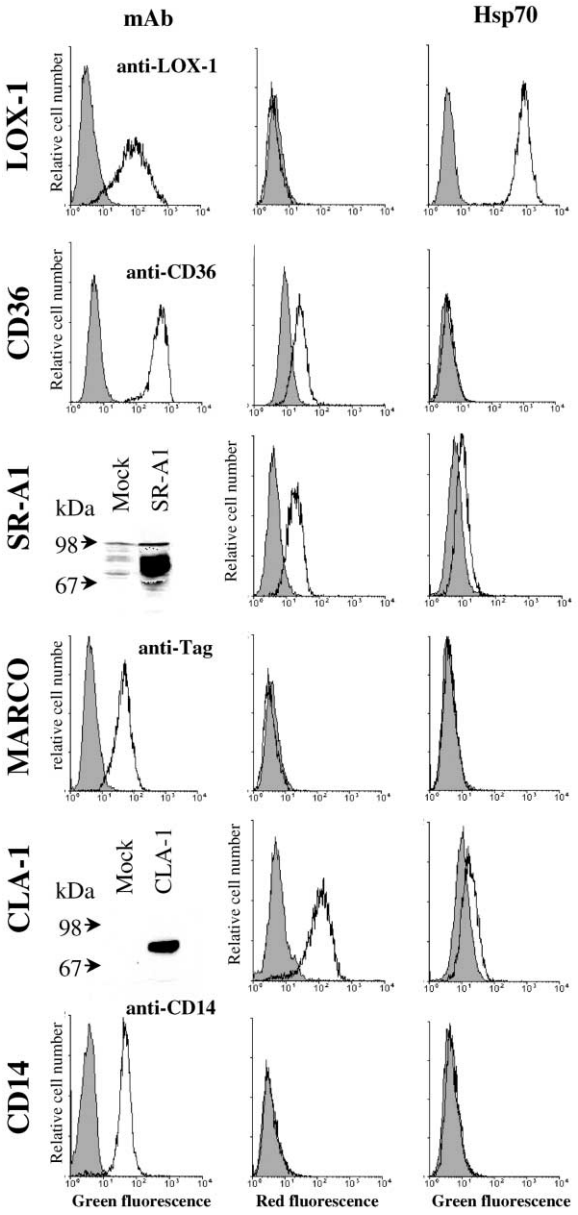

Figure 2. Hsp70 Binds to Recombinant LOX-1

CHO cells were transfected with the pEBS vector containing the cDNA encoding for the scavenger receptors LOX-1, CD36, SR-A1, MARCO, and CLA-1 or CD14. The expression of the SR was evaluated either by Western blotting (using an anti-tag protein C mAb as detection Ab) for SR-A1 and CLA-1 or by FACS for LOX-1, CD36, and MARCO (using 23C11, anti-CD36 mAb, or an anti-tag protein C mAb, respectively—bound mAbs were detected with Alexa^{488}-labeled anti-mouse Ig [left panel]). CD14 expression was verified using Alexa^{488}-labeled anti-CD14 mAb. Binding of fluorescent DiI-Ac-LDL (middle panel) and biotinylated Hsp70 (100 nM) detected with Alexa^{488}-labeled streptavidin (right panel) was evaluated by FACS. Gray histrograms correspond to unlabeled transfected cells.

Involvement of Scavenger Receptors in Hsp70 Binding to Dendritic Cells

Maleylated BSA (mBSA) is a ligand for numerous scavenger receptors (SR) (Abraham et al., 1995). Surprisingly, mBSA but not BSA prevented Hsp70 binding to peripheral blood myeloid DC (Figure 1C), and, to a lower extent, to macrophages (80 ± 10% and 55 ± 9% inhibition, respectively; mean ± sd, n = 5) (Figure 1D and Table 1). SR are cell-surface glycoproteins that bind modified lipoproteins and a broad spectrum of structurally unre-

lated ligands such as modified LDL (Ox- and Ac-LDL) (Dhaliwal and Steinbrecher, 1999), apoptotic cells, and bacteria-derived cell wall components (i.e., LPS and lipotechoic acid) (Krieger, 1999; Gough and Gordon, 2000). Inhibition assays were then extended to other ligands of SR. Oxidized-LDL, Ac-LDL, apoliprotein B (apoB), poly[I], and fucoidan, but not poly[A] (used as a negative control), also significantly prevented Hsp70 binding to DC, macrophages (Table 1), and monocytes (data not shown). The binding of Hsp60 and Hsp90 to human DC was also inhibited by mBSA (85 ± 10 and 72 ± 9% inhibition, respectively). These results suggest that SR could be cell surface binding elements for HSP on human APC.

We then analyzed the relative role of CD91 versus SR in Hsp70 binding to human macrophages and DC by evaluating the inhibitory activity of α2M and mBSA (ligands of CD91 and SR, respectively). Maleylated BSA was more potent than α2M in inhibiting Hsp70 binding to peripheral blood myeloid DC (80 ± 10 and 27 ± 5% inhibition using 2.5 μM of the compounds, respectively; mean ± sd, n = 5) (Figure 1D and Table 1). In contrast, mBSA and α2M inhibited Hsp70 binding to macrophages in a similar manner (55 ± 9 and 65 ± 6% inhibition, respectively) (Figure 1D and Table 1). Transferrin (Figure 1D) and BSA (Table 1), used as control proteins, did not affect Hsp70 binding to DC and macrophages. These results are in agreement with a role for CD91 as one HSP binding structure on macrophages (Binder et al., 2000). They also suggest a major role for SR in Hsp70 binding to DC.

Binding of Hsp70 to Recombinant LOX-1

In order to identify the SR that bind Hsp70, CHO cells were transfected with cDNA encoding for different SR. The expression of the recombinant SR by transfected cells was verified by Western blotting or FACS when appropriate (Figure 2, left panel) as well as by analyzing the binding of DiI-Ac-LDL (Figure 2, middle panel). Results showed that biotinylated Hsp70 bound to LOX-1-transfected CHO cells (LOX-1-CHO) (MFI = 875 ± 69; mean ± sd, n = 5) but not to the other SR tested: CD36, SR-A1, MARCO, and CLA-1 (Figure 2, right panel). Nontransfected CHO cells bind neither Hsp70 nor fluorescent Ac-LDL (DiI-Ac-LDL) (data not shown). As controls, biotinylated TT and BSA do not bind to LOX-1-CHO cells (data not shown). LOX-1, initially identified as an Ox-LDL receptor, also binds four-stranded nucleic acids and phospholipids (Sawamura et al., 1997; Moriwaki et al., 1998). Unlabeled Hsp70, mBSA, and different ligands of SR (including Ox-LDL, fucoidan, apoB, and poly[I]) significantly inhibited Hsp70 binding to LOX-1-CHO cells but not the control molecules BSA and poly[A] (Table 1). Interestingly, Hsp60 and Hsp90 also bound to LOX-1-CHO cells (MFI = 950 ± 60 and 640 ± 40, respectively; mean ± sd, n = 5). These data demonstrate that the scavenger receptor LOX-1 is a binding structure for Hsp70.

Involvement of LOX-1 in Hsp70 Binding to Dendritic Cells

The expression of the protein LOX-1 was reported on endothelial cells, smooth muscle cells, fibroblasts, and monocyte-macrophages (Sawamura et al., 1997; Moriwaki et al., 1998). Results from FACS analysis using the anti-LOX-1 mAb 23C11 showed that human peripheral blood myeloid DC and macrophages express LOX-1 (MFI = 38 ± 6 and 40 ± 8, respectively; mean ± sd, n = 5) (Figure 3A, upper panel). LOX-1 expression is lower on monocyte-derived immature DC generated by culturing monocytes with IL-4 plus GM-CSF (MFI = 18 ± 7) and undetectable on T cells and peripheral blood plasmacytoid DC (data not shown). LOX-1 expression on DC disappears after 2 days' treatment with the maturation factor LPS (data not shown). Western blotting analysis, performed using the anti-LOX-1 mAb 5-2 (Sawamura et al., 1997), confirmed these data (Figure 3B). As a control, LOX-1 was detected on LOX-1- but not mock-transfected CHO cells (Figure 3B). In agreement with these results, LOX-1 mRNA was detected in human monocytes, macrophages, peripheral blood myeloid DC, B cells, and, as previously reported, in human endothelial cells (Sawamura et al., 1997), but not in T cells and mature DC (Figure 3C).

The anti-LOX-1 mAb 23C11 but not an isotype control mAb totally prevented Hsp70 binding to LOX-1-CHO but not mock-transfected CHO cells (90 ± 5 and 3 ± 4% inhibition, respectively; mean ± sd, n = 5) (data not shown). Interestingly, anti-LOX-1 mAb 23C11 also prevented Hsp70 binding to human DC and macrophages (45 ± 8% and 32 ± 9% inhibition, respectively) (Figure 4).

Together, these data show that human immature DC express LOX-1 and evidence LOX-1 as a receptor for Hsp70 on DC. Whereas inhibition of Hsp70 binding to DC was partial with 23C11, it was total with some SR ligands (such as mBSA), suggesting that, in addition to LOX-1, other member(s) of the growing SR family are expressed on immature DC and bind Hsp70.

Involvement of LOX-1 in Antigen Cross-Presentation

HSP mediate peptide re-presentation (Udono and Srivastava, 1993; Srivastava et al., 1994; Singh-Jasuja et al., 2000; Castellino et al., 2000) and also initiate MHC class I-dependent immune responses against a whole antigen coupled or fused to HSP (Suzue et al., 1997). We thus analyzed whether LOX-1 is involved in Hsp70-mediated antigen cross-presentation using the murine ovalbumin (Ova)-specific B3Z $CD8^+$ hybridoma cells which produce IL-2 upon stimulation with Ova p257-264 peptide-K^b complexes (Karttunen et al., 1992). Hsp70 binds to murine immature DCs (Table 2) and macrophages (data not shown), and both cell types express LOX-1 (as evidenced by FACS using the 23C11 mAb) (Figure 3A, lower panel). The neutralizing 23C11 mAb but not a control mAb also inhibits Hsp70 binding to murine DC and macrophages (45 ± 8 and 29 ± 5% inhibition, respectively; mean ± sd, n = 5) (data not shown). Murine DC were pulsed with Ova coupled to Hsp70 (Hsp70-Ova) and exposed to the B3Z T cell hybridoma. A 10^2-fold lower concentration of Ova is required when coupled to Hsp70 compared with soluble Ova alone to induce similar proliferation of the CTLL-2 cell line (Figure 5A). Ova treated with the coupling reagent induces similar proliferation to untreated Ova (data not shown). Hsp70-Ova-induced IL-2 production is inhibited by anti-LOX-1 mAb (58 ± 9% inhibition using 50 μg/ml 23C11) (Figure 5B, left panel). Maleylated BSA and, to a lower extent, α2M also prevent Hsp70-induced

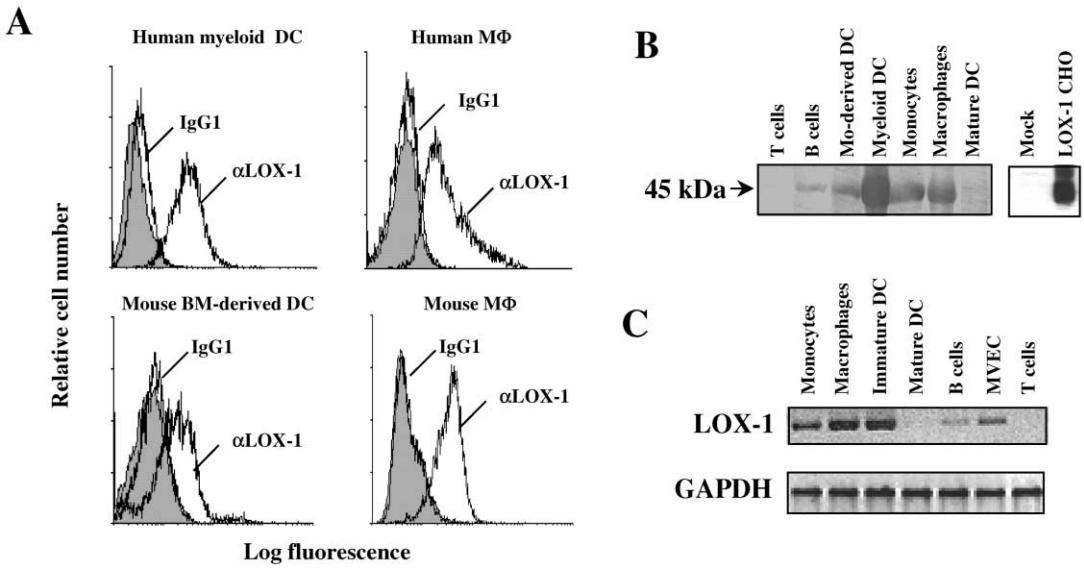

Figure 3. Hsp70 Binds to LOX-1

(A) Human and murine APCs express LOX-1. Human peripheral blood myeloid and monocyte-derived macrophages (Mϕ) and mouse bone marrow-derived DC and thioglycolate-elicited macrophages were incubated with 10 μg/ml Alexa^{488}-labeled anti-LOX-1 mAb 23C11 or isotype control IgG1 mAb and analyzed by FACS. Gray histogram corresponds to unlabeled cells.

(B) Human APCs express the protein LOX-1. Protein extracts from human monocytes, peripheral blood myeloid DC, monocyte-derived DC (Mo-derived DC), monocyte-derived macrophages, mature DC (LPS-treated peripheral blood myeloid DC), B cells, and T lymphocytes were analyzed for LOX-1 expression by Western blotting using the anti-LOX-1 mAb 5-2. Controls were LOX-1- and mock-transfected CHO cells.

(C) APCs express LOX-1 mRNA. The expression of LOX-1 mRNA was analyzed by RT-PCR in human monocytes, macrophages, immature and mature peripheral blood myeloid DC, B and T lymphocytes, and microvascular endothelial cells (MVEC). The amplified fragments were size-separated on a 1% agarose gel and visualized by ethidium bromide staining. RNA integrity and cDNA synthesis were verified by amplifying GAPDH cDNA.

Ova cross-presentation (75 $\pm$ 8% and 22 $\pm$ 6% inhibition using 2 μM of the compounds, respectively) (Figure 5B, right panel). Control IgG1 mAb and transferrin, used as negative controls, do not modulate Ova cross-presentation (Figure 5B). These data show that SR, and especially LOX-1, are involved in in vitro Hsp70-mediated cross-presentation of a whole antigen.

Targeting a Tumor Antigen to LOX-1 In Vivo Elicits a Protective Immune Response

We therefore evaluated whether targeting a tumor antigen to LOX-1 in vivo could elicit therapeutic and protective immune response against antigen-expressing tumor cells. We took advantage of the fact that 23C11 recognizes murine LOX-1 (Figure 3A, lower panel). C57BL/6 (H-2b) mice received s.c. injection of 2 $\times$ 10^4 E.G7 cells (a C57BL/6 thymoma transfected with Ova cDNA which is insensitive to NK cell-mediated lysis and to anti-Ova-mediated complement-dependent lysis; Storkus et al., 1989; Zhou et al., 1992) into the right flank and then injected three times (days 1, 10, and 20) with anti-LOX-1 mAb 23C11 coupled to Ova (anti-LOX-1-Ova) plus adjuvant. Results show that anti-LOX-1-Ova prevents tumor growth in 6/6 mice tested which remained tumor free after 40 days (Figure 5C). In mice injected with 23C11 alone or 23C11 plus Ova (not coupled), no significant delay in tumor growth was observed compared to mice injected with Ova alone (data not shown). In mice injected with isotype control IgG1 coupled to Ova (IgG1-Ova) plus adjuvant, tumor growth showed a slight delay compared to mice injected with Ova alone. Lastly, depletion in CD8$^+$ T cells prior to injection of anti-LOX-1-Ova abolished the protective effect of anti-LOX-1-Ova (data not shown). These results demonstrate that to target in vivo a tumor Ag to LOX-1 triggers a protective antitumor immune response.

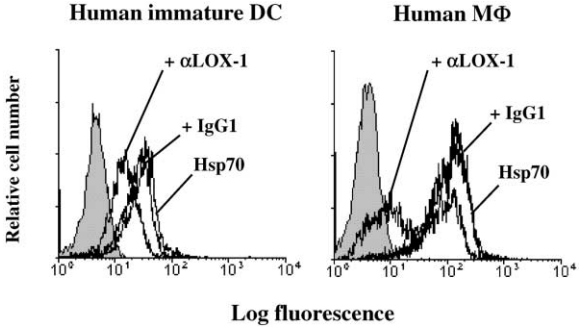

Figure 4. The anti-LOX-1 mAb 23C11 Inhibits Hsp70 Binding to Human APCs

Myeloid peripheral blood DC and macrophages were incubated with 50 μg/ml anti-LOX-1 or isotype control mAb prior to addition of 20 nM biotinylated Hsp70 followed by Alexa^{488}-labeled streptavidin.

Discussion

The antigen-presenting cells DCs and macrophages play a key role at the interface between innate and adaptive immunity. They express numerous receptors involved in the recognition and endocytosis of a large

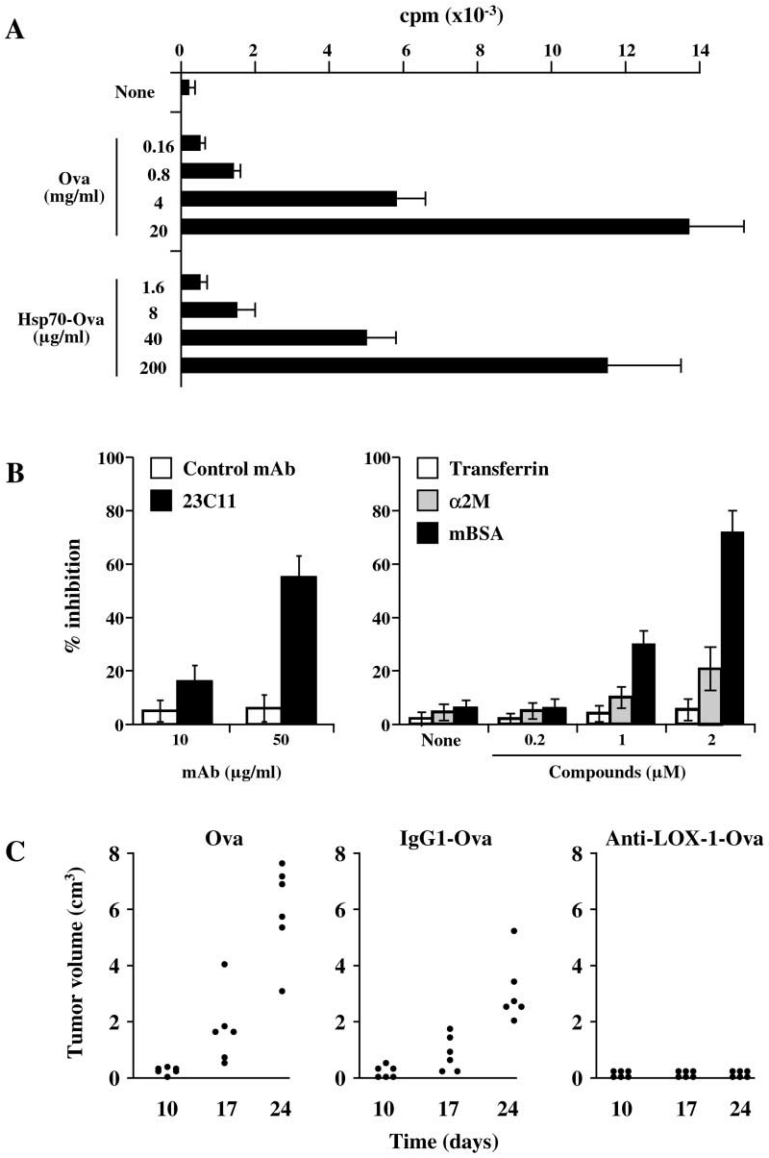

Figure 5. LOX-1 Mediates In Vitro and In Vivo Antigen Cross-Presentation

(A) Ova-Hsp70 is more potent than free Ova to activate the T cell hybridoma B3Z. Bone marrow-derived murine DC were pulsed for 8 hr with Ova alone, Ova treated with BS^3 (Ova-BS^3), or Ova coupled to Hsp70 (Ova-Hsp70) before being washed and recultured with B3Z T cells. The concentrations of Ova are indicated in the figure.

(B) Anti-LOX-1 mAb 23C11 prevents in vitro Hsp70-mediated Ova cross-presentation. Bone marrow-derived murine DC were pulsed for 6 hr with Ova-Hsp70 complex (corresponding to 10 μg/ml Ova) in the absence or presence of anti-LOX-1 mAb or control mAb (left panel) or in the presence of mBSA, α2M, or transferrin (right panel), at the indicated concentrations.

(A and B) IL-2 production was evaluated by measuring the proliferation of the IL-2-dependent cell line CTLL-2. Results are expressed in cpm (mean ± sd, n = 3) or as a percentage of inhibition of CTLL-2 proliferation.

(C) In vivo targeting of LOX-1 induces a protective antitumoral immune response. C57BL/6 (H-2b) mice received s.c. injection of 2×10^4 E.G7 cells into the right flank followed by 40 μg of Ova, IgG1-Ova, or anti-LOX-1-Ova split between both flanks at days 1, 10, and 20. The length (L) and width (W) was measured at different time points and tumor volume as $L \times W^2/2$.

number of pathogens, such as CD36, CLA-1, and the mannose receptor (Medzhitov and Janeway, 2000). While macrophages are specialized in ingesting and killing as many pathogens as possible, DC are the only APCs able to prime naive $CD4^+$ and $CD8^+$ T cells and to initiate immune responses (Cella et al., 1997; Banchereau et al., 2000).

The process by which some exogenous molecules (such as HSP, OmpA, and some toxins) (Goletz et al., 1997; Binder et al., 2000; Jeannin et al., 2000) are endocytosed by APCs via specific receptors, gain access to the MHC class I pathway, and stimulate $CD8^+$ cytotoxic T cells is called cross-presentation. The nature of the receptors expressed on DC and involved in antigen cross-presentation remains undetermined. Their identification is of great importance to initiate tumor-specific cytotoxic responses. We show here that SR, and among them LOX-1, are HSP binding structures expressed on DC and involved in the in vivo generation of protective CTL responses.

Previous studies reported that CD91 (α2 macroglobulin receptor) is a binding structure for HSP on macrophages and is involved in HSP-chaperoned peptide representation (Binder et al., 2000; Basu et al., 2001). We failed to detect CD91 on human DC, suggesting the existence of HSP binding structures other than CD91 on DC. Scavenger receptors bind ligands such as modified lipoproteins (Abraham et al., 1995), apoptotic cells, and bacteria-derived cell wall components and maleylated antigen (Dhaliwal and Steinbrecher, 1999; Krieger, 1999). We report that mBSA inhibits HSP binding to DC and macrophages. While mBSA and α2M inhibited Hsp70 binding to macrophages in a similar manner, mBSA mainly inhibited Hsp70 binding to immature DC. In agreement with these results, Basu et al. (2001) showed that α2M prevents the HSP-mediated peptide re-presentation by the murine macrophage cell line RAW264.7. Together, these data show that the relative role of CD91 versus SR in the binding of HSP varies with the nature of the APC. They also suggest that SR

are the main Hsp70 binding structures on DC while both CD91 and SR appear involved in Hsp70 binding to macrophages. Different authors failed to totally inhibit the binding of labeled Hsp70 to APCs with unlabeled Hsp70 (Sondermann et al., 2000; Habich et al., 2002; Lipsker et al., 2002). This result could be explained by the properties of scavenger receptors. Membrane-bound ligands are poorly displaced by ligands, a mechanism related to the association of SR-ligand complexes with microvilli or ruffles. Moreover, SR are rapidly internalized following ligand binding and are not subjected to down-regulation (Zha et al., 1997).

We show that HSP bind to the scavenger receptor LOX-1. LOX-1 was detected on endothelial cells, smooth muscle cells, fibroblasts, and monocyte/macrophages (Draude et al., 1999). Our results indicate that LOX-1 is also expressed on immature DC. In agreement with Western blotting analysis showing different isoforms of LOX-1 (probably related to differences in posttranslational modifications; Kataoka et al., 2000), we report that the anti-LOX-1 mAb reveals a broad immunoreactive band (particularly on DC and macrophages). LOX-1, initially identified as an Ox-LDL receptor (Asea et al., 2000), also binds four-stranded nucleic acids and phospholipids (Sawamura et al., 1997) and is involved in apoptotic cell phagocytosis via the recognition of phosphatidyl serine (Oka et al., 1998). The observation that compounds that inhibit Ox-LDL binding to LOX-1 totally prevent Hsp70 binding to LOX-1-transfected cells suggests that the binding site(s) of Hsp70 and Ox-LDL on LOX-1 are related or similar. Interestingly, the binding of Hsp70 to immature DC was totally inhibited by maleylated antigen and only partly inhibited by anti-LOX-1 mAb, suggesting that other members of the growing SR family could be involved in Hsp70 binding to APCs. The identification of these molecules is under investigation.

Previous studies reported the involvement of CD14 (Asea et al., 2000; Kol et al., 2000), of the complex TLR4/MD2 (Ohashi et al., 2000; Vabulas et al., 2002; Bulut et al., 2002), and of TLR2/TLR4 (Asea et al., 2002) in HSP-mediated cell activation. We show that LOX-1 is an Hsp70 binding structure expressed on DC. However, we failed to stimulate APC via LOX-1 (data not shown). Whether CD91 is a signaling receptor also remains unclear (Srivastava, 2002). Together, these data suggest that, in response to HSP, both endocytic (LOX-1 and CD91) and signaling (CD14, TLR2, and TLR-4) receptors may cooperate to activate APC. This hypothesis is supported by recent data obtained with yeast (Underhill et al., 1999) and OmpA (Jeannin et al., 2000). Additional experiments are required to determine the interactions between endocytic and signaling receptors in HSP-mediated cell activation.

HSP allow the cross-presentation of chaperoned peptides and coupled antigens. In order to analyze the role of LOX-1 in this pathway, we used ovalbumin coupled to Hsp70 (Suzue et al., 1997) to activate the T cell hybridoma B3Z, a model in which elicitation of $CD8^+$ T cells by a fusion protein is not dependent on the chaperone properties of the HSP (Huang et al., 2000). We show that LOX-1 is involved in the MHC class I-dependent presentation of Hsp70-associated Ag. Thus, in addition to having a role in lipid metabolism, SR may play a major role in antigen cross-presentation. The involvement of SR in cross-presentation has been previously suggested. Maleylation of an antigen results in both MHC class I- and class II antigen presentation in vitro (Abraham et al., 1995; Bansal et al., 1999). In addition, CD36 is involved in the uptake and cross-presentation of apoptotic bodies by DCs (Albert et al., 1998). Finally, SR-ligand complexes are endocytosed, a mechanism already reported for HSP and required for MHC class I presentation.

In addition to SR, CD91 has been reported to mediate antigen cross-presentation by macrophages (Binder et al., 2000; Basu et al., 2001). *Pseudomonas* exotoxin A (PEA), which targets antigens to the MHC class I pathway (Donnelly et al., 1993), binds to CD91 on macrophages (Kounnas et al., 1992). Thus, the observation that the two HSP binding structures identified so far (CD91 and LOX-1) are members of two related multiligand lipoprotein receptor families is in agreement with the hypothesis that innate cells have evolved to recognize a broad spectrum of danger signals with a restricted number of structures (Banchereau et al., 2000; Matzinger, 1994; Medzhitov and Janeway, 1997).

We report that targeting tumor antigen to LOX-1 in vivo induces the development of a protective antitumor $CD8^+$ T cell response. Moreover, mice were resistant to a second injection of tumor cells, suggesting the development of a memory T cell response (data not shown). As previously reported for mycobacteria Hsp70 (Suzue et al., 1997), Ova has to be covalently coupled to the anti-LOX-1 mAb to generate a protective anti-Ova T cell response. This observation suggests that the anti-LOX-1 mAb acts as a vector to target Ova to the receptor LOX-1. Injection of IgG1-Ova induced a slight delay in tumor growth compared to mice injected with Ova. Previous studies reported that FcγR mediates antigen cross-presentation of Ig-antigen complexes in vitro (Regnault et al., 1999) and primes antigen-specific $CD8^+$ CTL responses in vivo (Schuurhuis et al., 2002). This may contribute to explain why IgG1-Ova initiates a slight but significant protective anti-Ova T cell response. In vitro and in vivo data show that LOX-1 is one receptor involved in the trafficking of exogenous antigen toward the MHC class I pathway. In conclusion, we demonstrate that the scavenger receptor LOX-1 is expressed on DC and is a receptor that triggers the cross-presentation of whole antigens and the initiation of a protective antitumor immunity. We show that to target scavenger receptors, and especially LOX-1, is a novel approach to initiate in vivo protective MHC class I-dependent immune responses.

Experimental Procedures

Cell Isolation

Cells were cultured in RPMI 1640 medium supplemented with 10% FCS, 2 mM L-glutamine, and antibiotics (cRPMI) (all from Life technologies, Cergy Pontoise, France). Human BDCA-1^+ ($CD1c^+$), $CD11c^{high}$, and $CD123^{low}$ blood myeloid dendritic cells (Dzionek et al., 2000) were isolated using magnetic beads following instructions (Miltenyi Biotech, Bergish Gladbach, Germany). Human monocytes were isolated from peripheral blood mononuclear cells (PBMC) with anti-CD14 mAb-coated magnetic beads (Miltenyi Biotech). Macrophages were generated by culturing monocytes for 5 days in cRPMI containing 2 ng/ml GM-CSF plus 20 ng/ml M-CSF (R&D Systems, Abingdon, UK). Immature monocyte-derived dendritic cells were

generated by culturing monocytes with 20 ng/ml IL-4 plus 20 ng/ml GM-CSF (R&D Systems). In some experiments, human monocyte-derived DC and peripheral blood myeloid DC were treated with 10 ng/ml LPS (from *Escherichia coli* isotype O111:B4; Sigma, St. Louis, MO) for 2 days to induce DC maturation. All the experiments using human DC were performed with freshly isolated $CD11c^+$ myeloid peripheral blood DC, except Western blotting analysis that also presented LOX-1 expression in monocyte-derived DC. T cells were purified from peripheral blood mononuclear cells (PBMC) by roseting with sheep red blood cells, and B cells were purified by FACS-sorting the $CD19^+$ cells. Myeloid mouse immature DC were generated by culturing bone marrow cells from C57BL/6 ($H\text{-}2^b$) mice (Iffa Credo, L'Arbresle, France) for 6 days with 3 ng/ml murine GM-CSF (R&D Systems).

Proteins

Recombinant Hsp70 (StressGen Biotechnologies Corp, Victoria BC, Canada) and TT (SBL vaccine, Stockholm) were biotinylated using a commercial kit (Pierce, Rockford, IL). In order to remove contaminating endotoxin, biotinylated Hsp70 was passed through a column containing polymixin B coupled to resin (Pierce). The endotoxin level, determined by the limulus assay (Chromogenix, Milano, Italy), was less than 0.5 EU/mg biotinylated Hsp70. The cDNA sequences corresponding to the extracellular domain 71-273 of human LOX-1 was inserted in the expression vector Signal pIg-Tail (R&D Systems) in frame with the murine Fcγ1 (LOX-1-muFc). The fusion proteins were produced in PEAK cells (EdgeBio systems, Gaithersburg, MD) cultured in Optimem medium (Life technologies), and purified using protein A immobilized on agarose (Pierce).

Transfectants

The cDNA encoding for CD14 and the scavenger receptors CD36, MARCO, SR-AI, LOX-1, and CLA-1 were subcloned in the expression vector pEBS/PL containing a sequence encoding the protein C tag (EDQVDPRLIDGK) in frame with the inserted sequence. CHO cells (ATCC, Manassas, VA) were transfected using Fugene-6 (Roche Diagnostic, Meylan, France) and cultured in ISCOVE medium supplemented with 10% FCS and selected with hygromycin (all from Life Technologies) before clones were picked. Transfectants were selected as followed: CD14- and CD36-expressing cells were sorted by FACS using FITC-labeled anti-CD14 and anti-CD36 mAb (BD Pharmingen, San Diego, CA), respectively; LOX-1-expressing cells were isolated by FACS sorting using the anti-LOX-1 mAb 23C11 (see above); MARCO-, CLA-1-, and SR-AI-expressing cells were isolated by FACS sorting using fluorescent DiI-Ac-LDL (Harbor Bioproducts, Strougthon, MA). The expression of the SR was verified by FACS or by Western blotting when appropriate. CD14, CD36, and LOX-1 expression was analyzed by FACS using specific mAbs. MARCO expression was analized by FACS using anti-tag protein detected with FITC-labeled anti-mouse Ig mAb (Silenus, Melbourne, Australia). Isotype control mAbs were from BD Pharmingen. Results are expressed in MFI after subtraction of the MFI obtained with the isotype control mAb. SR-A1 and CLA-I expression was evaluated by Western blotting. In brief, cells were lysed in 10 mM phosphate buffer (pH 7.4) containing 0.5% Nonidet P40 (Sigma) and protease inhibitors (Boehringer Mannheim, Mannheim, Germany). Proteins from 5×10^6 cells were electrophoretically separated on a 10% polyacrylamide gel in nonreducing conditions and then transferred to a nitrocellulose membrane (Biorad, Ivry sur Seine, France). After saturation, membranes were incubated with an anti-protein C mAb (Roche Diagnostics). After washing, membranes were incubated with peroxydase-labeled anti-mouse IgG Ab (Dako, Glostrup, Denmark) and bound Abs were detected using the ECL system (Amersham Biosciences, Uppsala, Sweden).

Generation of an Anti-Human LOX-1 mAb

The anti-LOX-1 mAb 23C11 was produced by immunizing BALB/c mice (Iffa Credo) intraperitoneally with 100 μg of LOX-1-muFc in complete Freund's adjuvant. A second immunization was performed with incomplete Freund's adjuvant. Four days after a further intravenous inoculation of 50 μg/ml LOX-1-muFc, mice were killed and spleen cells were fused with SP2/O-Ag14 myeloma cells. After screening by FACS using LOX-1-CHO cells and nontransfected CHO cells used as control, the 23C11 clone was expanded and used for ascites fluid production. The 23C11 mAb was purified using protein A/G column (Pierce). The 23C11 mAb was used for FACS analysis and neutralizing experiments.

Binding Experiments

Biotinylated Hsp70 was incubated at 4°C for 30 min in FACS buffer (PBS containing 0.1% BSA and 0.05% NaN_3) and then washed in FACS buffer. Cells were then incubated with FITC-labeled streptavidin (Molecular Probes, Eugene, OR) for 20 min at 4°C before washings with FACS buffer. Fluorescence was analyzed using a FACScan (BD Biosciences, Franklin Lakes, NJ). In some experiments, cells were preincubated with unlabeled Hsp70, Hsp90, BSA, maleylated BSA (mBSA), polyinosinic acid (poly[I]), polyadenosic acid (poly[A]) (all from Sigma), Ox-LDL, or Ac-LDL (both from Biogenesis, Poole, UK) for 20 min at 4°C before incubation with biotinylated Hsp70. Bound Hsp70 was detected as described above.

Analysis of LOX-1 mRNA Expression by RT-PCR

The expression of the mRNA encoding for LOX-1 in professional APCs compared with microvascular endothelial cells (MVEC) was evaluated by RT-PCR. In brief, cells were resuspended in 1 ml Trizol reagent (Life Technologies). After extraction with chloroform, total RNA was precipitated with isopropyl alcohol. Single-strand cDNA was synthesized using 2 μg of total RNA by reverse transcription using an oligo-dT primer and reverse transcriptase (Promega, Madison, WI). PCR amplification was performed with an amount of cDNA corresponding to 25 ng of starting total RNA (2 min at 94°C followed by 30 cycles (30 s at 94°C, 1 min at 60°C, and 1 min at 72°C) followed by a final extension of 4 min at 72°C) using specific oligonucleotides (5′-ACTGGAGGGACAGATCTCAGCCGG-3′ and 5′-GGAAATTGCT TGCTGGATGAATCC-3′). RNA integrity was assessed by GAPDH cDNA amplification. The PCR products were analyzed on a 1% agarose gel by electrophoresis in the presence of ethidium bromide.

Analysis of LOX-1 Expression by Human APCs

The expression of LOX-1 in monocytes, immature DC, macrophages, B cells, and T lymphocytes was evaluated by FACS and Western blotting. Controls included LOX-1-CHO cells and nontransfected cells. LOX-1 expression was analyzed by FACS using $Alexa^{488}$-labeled anti-LOX-1 23C11 mAb. Results are expressed in MFI after subtraction of the MFI obtained with the control mAb. For Western blotting, after cell lysis, proteins were separated by SDS-PAGE in a 10%–20% polyacrylamide gradient gel and transferred to a PVDF membrane. The membrane was probed with the anti-LOX-1 antibodies #5-2 (Sawamura et al.,1997). Bound Abs were visualized using the avidin-biotin system (Vector, Burlingame, CA) and Konica Immunostaining kit (Konica, Mahwah, NJ).

Activation of the Ova-Specific T Cell Hybridoma B3Z

B3Z T cell hybridoma cells are specifically activated by the Ova-derived peptide SIINFEKL presented by $H\text{-}2K^b$. Ovalbumin (Ova) (Worthington, Lakewood, NJ) was coupled to Hsp70 (Ova-Hsp70) using bis(sulfosuccinimidyl)suberate (BS^3) used at 0.25 mM as described by the manufacturer (Pierce). As a control, Ova was treated with BS^3 (Ova-BS^3). Proteins and conjugates were assayed with the BCA kit (Pierce). The quantity of coupled Ova was determined by ELISA using specific anti-Ova Abs. Immature DC/macrophage precursors were generated as previously reported and pulsed for 8 hr in FCS free medium with Hsp70, Ova alone, Ova-BS^3, or Ova-Hsp70. After washings, 5×10^5 pulsed DC were cultured with 5×10^5 B3Z cells. In some experiments, DC were incubated with Ova-Hsp70 in the presence of a 2M, mBSA, transferrin, anti-LOX-1 mAb 23C11, or control mAb. IL-2 production in the 16 hr supernatants was evaluated by measuring the proliferation of the IL-2-dependent cell line CTLL-2 (ATCC).

Treatment of Established Tumors

Groups of six mice received s.c. injections of 1.5×10^4 E.G7 cells ATCC) into the right flank followed by 100 μg of Ova, Ova coupled to anti-LOX-1 mAb 23C11 (anti-LOX-1-Ova) or to a control mAb (IgG1-Ova) (Sigma) split between both flanks with incomplete Freund's adjuvant at days 1, 10, and 18. The tumor length (L) and

width (W) was measured at different time points, and tumor volume was determined by $L \times W^2/2$.

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