Escherichia coli Expressing Recombinant Antigen and Listeriolysin O Stimulate Class I-Restricted CD8^{+} T Cells following Uptake by Human APC[1]

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Vaccination against cancer or intracellular pathogens requires stimulation of class I-restricted CD8^{+} T cells. It is therefore important to develop Ag delivery vectors that will promote cross-presentation by APCs and stimulate appropriate inflammatory responses. Toward this goal, we tested the potential of *Escherichia coli* as an Ag delivery vector in in vitro human culture. Bacteria expressing enhanced green fluorescent protein were internalized efficiently by dendritic cells, as shown by flow cytometry and fluorescence microscopy. Phenotypic changes in DC were observed, including up-regulation of costimulatory molecules and IL-12p40 production. We tested whether bacteria expressing recombinant Ags could stimulate human T cells using the influenza matrix protein as a model Ag. Specific responses against an immunodominant epitope were seen using IFN-γ ELISPOT assays when the matrix protein was coexpressed with listeriolysin O, but not when expressed alone. THP-1 macrophages were also capable of stimulating T cells after uptake of bacteria, but showed slower kinetics and lower overall levels of T cell stimulation than dendritic cells. Increased phagocytosis of bacteria induced by differentiation of THP-1 increased their ability to stimulate T cells, as did opsonization. Presentation was blocked by proteasome inhibitors, but not by lysosomal protease inhibitors leupeptin and E64. These results demonstrate that recombinant *E. coli* can be engineered to direct Ags to the cytosol of human phagocytic APCs, and suggest possible vaccine strategies for generating CD8^{+} T cell responses against pathogens or tumors. *The Journal of Immunology,* **2004, 172: 1595–1601.**

Effector CD8^{+} T cells lyse targets such as virally infected cells or tumors via recognition of endogenously synthesized peptide Ags bound to class I MHC proteins (1). Generation of CD8^{+} T cells requires initial activation by APCs, primarily dendritic cells (DC)[3] that typically do not synthesize the Ag, but instead acquire it from extracellular sources. The process of cross-presentation, involving uptake of Ag from exogenous sources, appears essential for stimulating CD8^{+} T cell responses against most viruses, intracellular parasites and bacteria, and potentially tumor cells (2–7).

How DC take up and process Ags for MHC class I presentation has been studied intensively recently, but is still not completely understood. A critical step appears to involve export of Ag, probably in partially processed form, from an endocytic compartment into the cytosol, in which further processing by proteasome and translocation by TAP into the endoplasmic reticulum lumen occurs (8). It has been demonstrated using Ag-Ab complexes internalized via Fc receptors that processing of Ag into fragments smaller than 30 kD in size facilitates their export from endosomes (9). Other means for translocating Ag into the cytosol are present in some intracellular bacteria, such as *Listeria*, that contain the pore-forming hemolysin listeriolysin O (LLO) (10, 11). LLO is sufficient to allow for MHC class I presentation of Ag when coexpressed in *Escherichia coli* that are phagocytosed by APC such as macrophages and DC (12).

The latter experimental system, developed initially by Higgins et al. (12) using a mouse model involved coexpression of OVA with LLO in *E. coli*, which following uptake by APC, can be recognized by the OVA-specific B3Z hybridoma in vitro (12, 13). Coexpression of OVA and LLO enhanced presentation by four orders of magnitude when compared with OVA alone. More recently, it was shown that a B16 OVA-expressing tumor could be rejected in mice following immunization with *E. coli* expressing both OVA and LLO (14).

In the current study, we have tested whether *E. coli* expressing an immunodominant pathogen-derived epitope together with LLO could stimulate Ag-specific human T cells. The Ag tested was the amino acid residues 58–66 epitope of human type A influenza virus matrix protein (M1 protein) that binds to HLA-A2 (15–20). We observed that T cell stimulation was dependent on LLO coexpression, and also on proteasome activity. This is the first report documenting that LLO-enhanced cross-presentation occurs in a human cell system, with a pathogen-derived peptide and primary human T cells. This supports the possibility that vaccines targeting enhanced CD8^{+} T cell responses might be developed by manipulating Ag delivery pathways within APC.

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[3] Abbreviations used in this paper: DC, dendritic cell; EGFP, enhanced fluorescence protein; LLO, listeriolysin O; M1, influenza matrix protein; PSI, proteasome inhibitor I; PFA, paraformaldehyde; BCG, bacille Calmette-Guérin.

Materials and Methods

Recombinant E. coli and proteins

E. coli strain TOP 10 was purchased from Invitrogen (Carlsbad, CA) and used for plasmid amplification. *E. coli* strain BL21DE3 competent, which contains inducible T7 polymerase, was purchased from Novagen (Madison, WI) for protein expression.

The cDNA of type A influenza matrix protein (M1 protein) was amplified from a type A influenza cDNA by PCR using primers with *Nde*I sites in overhangs and a stop codon in the downstream primer. The 5′ primer used was 5′-GGGATTCATATGAGTCTTCTAAC-3′. The 3′ primer used was 5′-GCTAAGTTCACTGGGTATACTTAGGG-3′. The PCR product was cloned using TOPO TA Cloning kit (Invitrogen). The sequence of the PCR product was analyzed by restriction enzyme digestion and automated DNA sequencing, and shown to be identical to the previously published M1 sequence (21, 22).

To construct the M1 protein expression vector, the M1 cDNA was subcloned into the *Nde*I site of pET22b$^+$ vector (Novagen), in front of the "leading" sequence of the multiple cloning sites. Therefore, the M1 protein was designed to be expressed without any vector-derived sequences, such as the leader sequence or the His-tag pET22b. This construct, designed to express M1 protein, is designated as pET22b.M1 throughout the study. The correct orientation of the cDNA was determined by analysis of restriction enzyme digests (data not shown). To express the M1 protein, pET22b.M1 was transformed into *E. coli* strain BL21DE3. The enhanced green fluorescence protein (EGFP) was expressed in an expression vector construction using pET15 as backbone (designated pET15.EGFP), was a kind gift from Dr. A. Gambotto (University of Pittsburgh School of Medicine, Pittsburgh, PA), and was also expressed in BL21DE3. Plasmid pDP3615, which carries the *hly* gene encoding LLO lacking its secretion signal sequence, under the regulation by a constitutive *tet* gene promoter (12), was transformed into *E. coli* BL21DE3 carrying either pET22B.M1 or pET15.EGFP. Coexpression of the LLO with M1 protein or EGFP was achieved essentially the same as previously described by Higgins et al. (12). Dual plasmids in *E. coli* BL21DE3 were maintained in medium containing 100 μg/ml of ampicillin and 50 μg/ml of chloroamphenicol. Isopropyl β-D-thiogalactoside was added to a final concentration of 300 μM for the final 2 h to induce protein expression.

Western blot

For the detection of M1 protein expression, *E. coli* were lysed directly in SDS sample buffer and resolved by SDS PAGE. Proteins were transferred to a 0.45-μm nitrocellulose membrane, which was incubated with 1/20,000 diluted Ab 2BB10-G9 (23), a mAb against the type A influenza matrix protein (a gift from Dr. D. Bucher, New York Medical College, Valhalla, NY). After incubation with the primary Ab, the membrane was incubated with a 1/2000 diluted HRP-conjugated goat anti-mouse Ab. The membrane was developed by adding a chemoluminescent substrate, SuperSignal West Femto Substrate (Pierce, Rockford, IL) and exposed to x-ray film.

Ag-presenting cell isolation and culture

DCs were generated from CD14$^+$ peripheral blood monocytes, and were cultured in IMDM medium containing GM-CSF and IL-4 as previously described (24). Briefly, PBMCs were purified by standard Ficoll-Paque (Amersham Pharmacia, Uppsala, Sweden) gradient centrifugation of buffy-coats obtained from healthy blood donors. Monocytes were obtained by 1-h adherence of PBMC in IMDM supplemented with 10% FBS and followed with five washes of HBSS. GM-CSF and IL-4 were each added at a concentration of 1000 U/ml (24). DCs were used at day 5 in our experiments, unless specifically mentioned. As shown previously, these DCs have an immature phenotype with low to moderate expression of MHC class I and class II, CD40, CD80, CD83, and CD86.

T2 cells, which are HLA-A*0201$^+$ and TAP-deficient, were maintained in RPMI 1640 medium. THP-1 cells, obtained from Dr. O. Finn (University of Pittsburgh School of Medicine), are an HLA-A*0201$^+$ monocytic cell line that can differentiate and acquire macrophage functions, such as phagocytosis, following treatment with a number of compounds. In our experiments, vitamin D$_3$ and retinoic acid were added to a final concentration of 0.1 μM ~16 h before experiments to induce differentiation.

Generation of human CD8$^+$ T cells specific for M$_{58-66}$ peptide

Leukapheresis research products were purchased from the Central Blood Bank (Pittsburgh, PA) as a source of PBMC. Anti-HLA class I mAbs were used to screen purified PBMC for expression of HLA-A2. HLA-A2$^+$ PBMC were used to generate a cell line specific for the influenza M$_{58-66}$ epitope as follows. Purified PBMCs were plated at the concentration of 2 × 10^6 per well in a 24-well tissue culture plate. During the initial 5-day
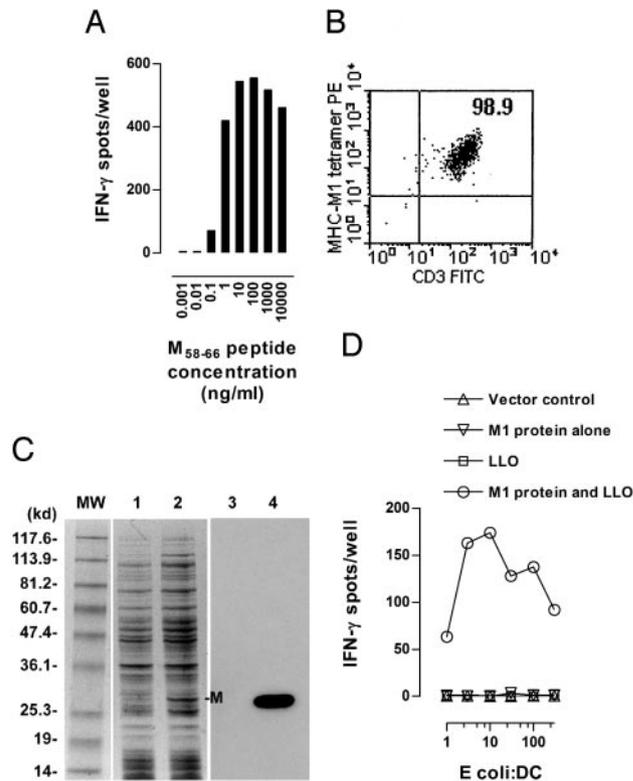
FIGURE 1. Recognition of M$_{58-66}$ epitope by human CD8$^+$ T cells. *A*, CD8$^+$ T cell line expanded from normal HLA-A2$^+$ blood donor responds to M$_{58-66}$ peptide pulsed onto T2 cells. ELISPOT assay was used to detect IFN-γ secretion by T cells following Ag stimulation. *B*, Tetramer staining of M1-specific T cell line using PE-labeled HLA-A2/M158-66 tetramer (*y*-axis) and FITC-labeled anti-CD3 (*x*-axis). *C*, Expression of M1 protein in *E. coli* strain BL21DE3 demonstrated after protein induction by isopropyl β-D-thiogalactoside followed by lysis and SDS-PAGE. Coomassie blue staining (*lanes 1* and *2*) or Western blotting (*lanes 3* and *4*) of samples from bacteria transformed with pET22b vector alone (*lanes 1* and *3*) or pET22b/M1 (*lanes 2* and *4*). The position of M1 on the gel (M) is indicated. For Western blotting, mAb 2BB10-G9 specific for M1 protein was used. Comparable analyses were performed to confirm LLO expression (our unpublished observations). *D*, M1-specific T cell stimulation following exposure of DC to *E. coli* expressing the indicated constructs at ratios indicated on the *x*-axis. Only coexpression of M1 protein and LLO resulted in cross-presentation detectable by ELISPOT.

culture, PBMCs were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated human AB serum in the presence of 1 μg/ml M$_{58-66}$ peptide. At day 6, PBMC cultures were refreshed with complete RPMI 1640 medium containing 20 U rIL-2. After the second restimulation with complete RPMI 1640 containing rIL-2 between day 9 and day 12, T cells were subjected to limiting dilution at 10, 30, and 100 cells per well in U-bottom shaped plates. T cell cultures were stimulated with 0.1 μg/ml PHA and 20 U/ml rIL-2 in RPMI 1640 medium containing heat-inactivated FBS. Three to 4 days after stimulation, cultures were refreshed with RPMI 1640 medium containing FBS and 20 U/ml rIL-2. T cells were restimulated with 0.1 μg/ml PHA plus 20 U/ml rIL-2 every 7–10 days. After 2–3 wk, growing wells were identified by microscopic inspection and transferred to a new plate. These wells were tested for Ag specificity using M$_{58-66}$ peptide pulsed T2 cells as APCs. Specific wells were identified and expanded by stimulating with PHA and irradiated allogeneic PBMCs.

Tetrameric MHC-peptide analysis of T cells

iTAg-tetramer specific for M$_{58-66}$-specific T cells was purchased from Beckman Coulter (San Jose, CA). To analyze binding, 2 × 10^5 T cells were resuspended in FACS buffer (5% FBS in PBS) and incubated on ice for 1 h with 2 μl tetramer and FITC-conjugated anti-CD3 Ab. Cells were washed once with 4 ml ice-cold FACS buffer and fixed with 200 μl of 2% paraformaldehyde (PFA). Samples were analyzed using a Becton Dickinson FACSCaliber (BD Biosciences, San Jose, CA).
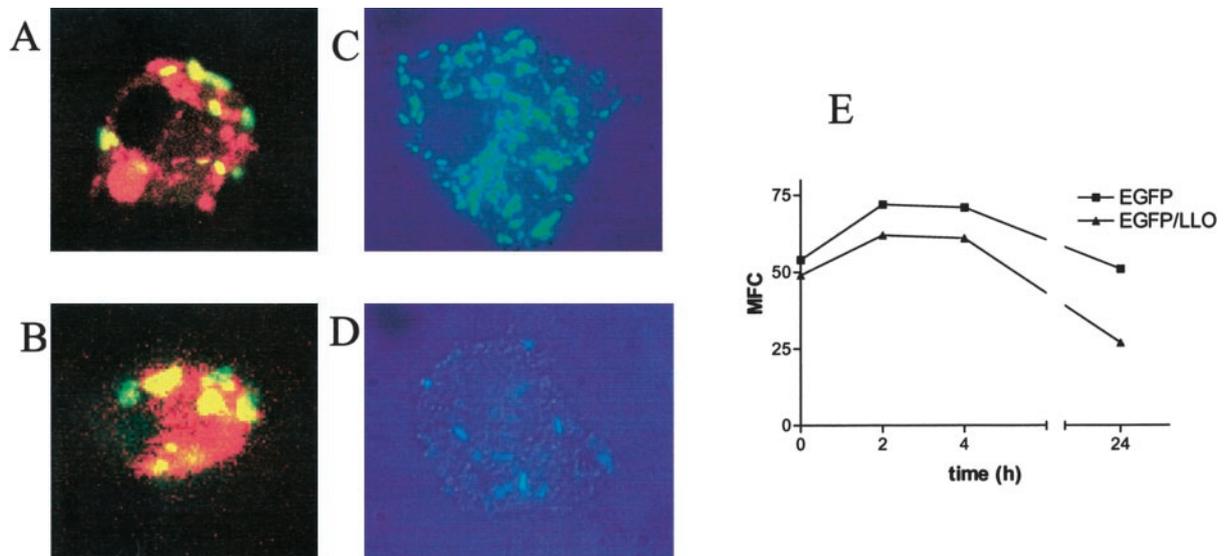
FIGURE 2. Disappearance of green fluorescence is accelerated by coexpression of EGFP and LLO in bacteria taken up by DC. *A* and *B*, Confocal microscopy of DC following exposure for 1 h to EGFP-expressing *E. coli*. Lysosomes (red) were labeled with LysoTracker, and bacteria (green) are also shown. Colocalization (yellow) is shown in these representative images. Comparable uptake was seen for bacteria expressing EGFP alone (*A*) or together with LLO (*B*). A single z-section is shown in each image. *C* and *D*, Incubation of bacteria for 24 h with DC followed by imaging using fluorescence and differential interference contrast imaging. Diminished green fluorescence is seen in bacteria coexpressing LLO and EGFP (*D*) compared with EGFP alone (*C*). *E*, Flow cytometry based assay for measuring fluorescence following uptake of bacteria by DC confirming reduced EGFP stability in bacteria expressing both proteins. This experiment was repeated three times and gave comparable results each time, showing ~50% reduction in fluorescence intensity by 24 h. MFC, mean fluorescence channel.

ELISPOT assay for IFN-γ production

Ninety-six-well nitrocellulose plates (MultiScreen-HA plate, MA-HAS4510; Millipore, Bedford, MA) were coated with 75 μl/well of 8 μg/ml mouse anti-human IFN-γ mab clone 1-D1K in PBS (Mabtech, Nacka, Sweden) overnight at 4°C. Plates were washed four times before addition of 200 μl RPMI 1640 plus 10% FBS for over 2 h at 37°C to block additional protein binding sites. M_{58-66}-specific T cells were then added at the concentration of 10,000 cells per well in a volume of 100 μl and 50,000 APCs were added into each of duplicate wells with gentamicin at the final concentration of 100 μg/ml. T cells for these assays were either taken directly from culture, or thawed and used immediately after washing. After 20 h incubation at 37°C in 5% CO$_2$, plates were developed by incubating with 75 μl of 2 μg/ml anti-IFN-γ biotinylated mAb 7-B6-1 (Mabtech) in PBS plus 0.5% BSA for 2 h at 37°C. Avidin-peroxidase complex (100 μl), Vectastain Elite kit (Vector Laboratories, Burlingame, CA) was added to each well after six washes with PBS plus 0.05% Tween 20 and incubated for 1 h at room temperature. The plate was developed using 100 μl of 3-amino-9-carbazole solution (AEC Staining kit; Sigma-Aldrich, St. Louis, MO). Typically, 5% of M_{58-66}-specific T cells produce positive spots upon stimulation with peptide-pulsed APCs. The number of spots were obtained using an Immunospot analyzer (Cellular Technology, Cleveland, OH) and typically varied by <10% for duplicate wells.

Incubation of either M_{58-66} peptide or bacteria with APCs

Either freshly cultured DCs, T2 cells, or THP-1 cells were harvested and resuspended in RPMI 1640 medium supplemented with 10% FBS at the concentration of 2 × 10^5 cells/ml. Either M_{58-66} peptide or *E. coli* was added to 10^5 APCs in a volume of 0.5 ml in 5 ml polypropylene tubes (cat. no. 352603; Falcon, Franklin Lakes, NJ) for 1 h. After a 1-h incubation, APCs were centrifuged, and Ag pulsing medium was decanted. APCs were washed with 2 ml RPMI 1640 supplemented with 10% FBS. After the final wash, APCs were mixed with T cells as previously described.

For fixation of cells, APCs were washed once with 2 ml PBS after Ag incubation then immediately fixed in 1 ml 0.5% PFA for 10 min on ice. At the end of PFA fixation, 2 ml of RPMI 1640 containing 5% FBS were added and APCs were collected by centrifugation. After two additional washes, APCs were added to an ELISPOT plate.

Ab coating of bacteria

Bacteria were preincubated with a polyclonal anti-*E. coli* rabbit antiserum as directed by the manufacturer (Molecular Probes, Eugene, OR). Recon-stituted antiserum (1 μl) was incubated with 3 × 10^9 bacteria for 30 min in a total volume of 100 μl PBS, before washing and resuspension in PBS. This amount of antiserum is designated 1×. Ten-fold concentration or dilutions of the serum were also tested.

Measurement of E. coli phagocytosis

THP-1 cells were grown overnight in 48-well plates (1 × 10^5 cells per well). Rabbit anti-*E. coli* polyclonal antiserum was added to washed bacteria for 30 min, and the bacteria were then washed twice in PBS. EGFP-expressing *E. coli* with or without opsonizing Ab were added to the THP-1 cells at dilutions of ~100/1. At various times, cells were collected and washed 2× in PBS, then fixed with 2% PFA before analysis by flow cytometry.

Treatment of cells with protease and proteasome inhibitors

Protease inhibitors leupeptin and E64 were obtained from Sigma-Aldrich. Lactacystin (clasto-lactacystin β-lactone) and proteasome inhibitor I (PSI) were obtained from EMD Biosciences (San Diego, CA) and MG132 from Calbiochem (San Diego, CA). Inhibition experiments were essentially the same as previously described, except that inhibitors were added to APCs at varying concentration 10 min before adding Ag. After 5 h continuous incubation with either peptide or *E. coli*, APCs were washed and fixed with 0.5% PFA. After fixation, APCs were mixed with T cells in an ELISPOT plate.

Results

Coexpression of influenza matrix protein and LLO in E. coli is required for cross-presentation to CD8$^+$ T cells

To analyze the requirements for cross-presentation of Ags delivered by bacterial vectors to human DCs, we chose an Ag that has been previously characterized in detail for both epitope specificity and host immune response. Amino acid residues 58–66 of M1 protein (M_{58-66}) bind to HLA-A2 subtypes present in ~50% of humans. The epitope is generated in influenza-infected cells and also in DCs after phagocytosis of apoptotic fragments of virus-infected cells (5). Circulating T cells specific for M_{58-66} are detectable in most previously exposed HLA-A2$^+$ individuals as

FIGURE 3. Level of bacterial phagocytosis correlates with Ag presentation capacity of THP-1 cells. *A*, THP-1 cells present M_{58-66} epitope to T cells when pulsed with M1/LLO-expressing *E. coli*. *B*, Treatment of THP-1 cells overnight with retinoic acid and vitamin D_3 enhanced presentation of M_{58-66} epitope processed from *E. coli* expressing M1 and LLO proteins. *C*, Exogenous M_{58-66} peptide is presented equally well by treated and untreated cells. *D* and *E*, Opsonization of *E. coli* using antibacterial antiserum enhances both uptake as determined by flow cytometric assay (*D*) and Ag presentation shown by IFN-γ ELISPOT (*E*). Assays were performed using bacteria expressing either EGFP alone (*D*) or M1 and LLO (*E*). For these experiments, use of the recommended amount of antiserum per quantity of bacteria as suggested by the manufacturer is designated as 1×. A 10-fold higher concentration or dilutions were also tested, as previously indicated using *x*-axes and described in *Materials and Methods*.
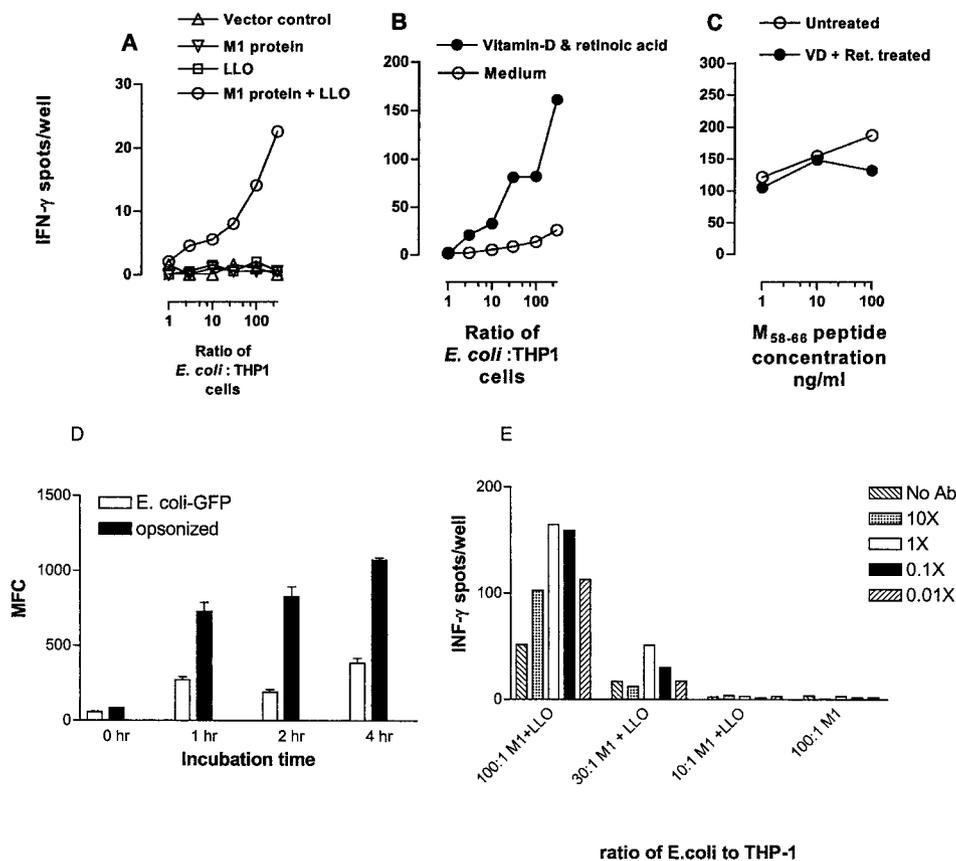
shown by both IFN-γ ELISPOT and tetrameric MHC-peptide analyses (15, 17, 19, 20).

T cells specific for epitope M_{58-66} were generated from a HLA-A2 healthy blood donor as described in *Materials and Methods*. The specificity of these T cells was determined by IFN-γ ELISPOT assay using M_{58-66} peptide-pulsed T2 cells as APC (Fig. 1*A*). M_{58-66}-specific T cells responded to as little as 0.1 ng/ml of M_{58-66} peptide. These T cells were essentially clonal as they were 98.9% positive when stained with a tetrameric HLA-A2 loaded with M_{58-66} peptide (Fig. 1*B*).

To test requirements for Ag cross-presentation in DC, DC from HLA-A2$^+$ individuals were incubated with bacteria expressing different protein Ags and then analyzed using M_{58-66}-specific T cells. Confirmation that recombinant proteins were expressed in bacteria was obtained by SDS-PAGE or Western blotting as shown in Fig. 1*C*. Following incubation of DC with M1-expressing *E. coli*, ELISPOT was used to measure IFN-γ secretion by T cells as a readout of function. As shown in Fig. 1*D*, bacteria expressing M1 protein alone did not stimulate T cell recognition, even at ratios of bacteria to DC as high as 300:1. In contrast, coexpression of M1 with LLO stimulated responses detectable at ratios as low as 1:1. This result is consistent with those reported in mouse systems developed by Higgins et al. (12) and Campbell and coworkers (13), showing that LLO greatly enhanced presentation of OVA-derived epitopes.

Localization of bacteria in DC following uptake using fluorescence-based assays

To address whether LLO-mediated differences in bacterial uptake or localization influenced subsequent processing and Ag presentation, we generated *E. coli* expressing either EGFP alone or together with LLO. Bacteria were incubated with DC and uptake measured using flow cytometric and fluorescence microscopy-

based assays. Both types of bacteria were internalized by DC and localized to lysosomes as determined using confocal microscopy (Fig. 2, *A* and *B*). Differences in EGFP stability were noted in time course experiments, however, suggesting either enhanced degradation or export of the protein from the endocytic compartment (Fig. 2, *C–E*). The latter might be explained by the ability of LLO to perforate the phagolysosomal membrane, leading to enhanced degradation in the cytosol. In addition, we tested the ability of LLO-expressing *E. coli* to induce maturation and observed no differences from bacteria expressing EGFP alone (our unpublished observations). IL-12p40 production as assayed by ELISA was also induced at similar levels in DC following exposure to LLO-expressing bacteria (our unpublished observations). Together, these latter results suggest that expression of LLO in *E. coli* does not alter their ability to induce phenotypic maturation and cytokine secretion in DC. In summary, LLO-expressing *E. coli* localize efficiently to phagolysosomes, and due apparently to decreased intracellular stability, may deliver Ags more efficiently into the cytosol of cells.

Phagocytosis is required to facilitate Ag cross-presentation

We next examined whether phagocytosis of bacteria was required for the delivery of Ag to the class I cross-presentation pathway. In prior studies in the mouse, this question was not directly addressed, as the cells used were actively phagocytic. Human THP-1 monocytes (HLA-A2$^+$) were analyzed, either with or without the addition of retinoic acid and vitamin D_3, which was shown previously to induce their differentiation into macrophages. We and others have shown that THP-1 cells stop proliferating, become adherent, and increase their phagocytic capacity after differentiation (our unpublished observations and Refs. 25, 26).

As with DC, Ag presentation by THP-1 was dependent on coexpression of LLO (Fig. 3*A*), although overall the efficiency was

lower. The presentation of M_{58-66} epitope from M1 plus LLO expressing *E. coli* was significantly increased after the treatment of THP-1 cells with vitamin-D₃ and retinoic acid whereas presentation of exogenously added peptide was not altered (Fig. 3, *B* and *C*). Phagocytosis of *E. coli* as measured by flow cytometric assay described in *Materials and Methods* increased ~5-fold, with the mean fluorescence channel increasing from 137 for untreated cells to 667 for treated cells. We next tested whether Ag presentation was increased following opsonization of bacteria with anti-*E. coli* antiserum. As seen in Fig. 3, *D* and *E*, both uptake of bacteria by THP-1 cells and T cell recognition were increased significantly following bacterial opsonization. These results support the conclusion that the level of phagocytosis is correlated with Ag processing and presentation.

Both live and antibiotic-killed E. coli *can deliver Ags for cross-presentation by DC*

Because LLO-mediated Ag cross-presentation is most likely dependent on the biochemical activity of the protein within the phagolysosome, we suspected that antibiotic-killed *E. coli* might also be able to deliver Ag for cross-presentation. Recombinant *E. coli* expressing both M1 protein and LLO were pretreated with gentamicin or kanamycin before being incubated with THP-1 cells. As shown in Fig. 4*A*, THP-1 cells incubated with either live *E. coli* or antibiotic-killed *E. coli* produced essentially the same response as measured by IFN-γ ELISPOT. We also tested whether PFA-fixed *E. coli* could induce presentation of the epitope. The response was significantly decreased with increased fixation time using 0.5% PFA (Fig. 4*B*).

Kinetics of Ag cross-presentation following E. coli-*based Ag delivery*

To measure the kinetics of the M_{58-66} epitope presentation, DC or THP-1 cells were removed at different time points after addition of bacteria, and fixed for 10 min in 0.5% PFA to terminate Ag processing. After the fixation, the cells were washed as described in *Materials and Methods* before addition with T cells to ELISPOT assays. Fig. 5 shows that DC can process and present Ag very rapidly, within 1–2 h after uptake, whereas THP-1 cells showed a slower kinetics, and were ultimately less efficient. Maximal presentation by DC was observed in this assay by 4 h, and similar

FIGURE 5. Time course of M_{58-66} presentation by DC and THP-1 cells. DC or THP-1 cells were pulsed with *E. coli* (1:10 ratio) expressing both M1 and LLO proteins for 1 h, washed, and incubation continued for the time indicated on the *x*-axis. Cells were then fixed with PFA, washed extensively, and incubated with M1-specific T cells for IFN-γ ELISPOT assay.

results were obtained in repeat experiments. In contrast, presentation increased for up to 10 h after addition of *E. coli* to THP-1 cells. It should be noted that T cell responses are lower using fixed instead of live cells as APC, as evident in this figure. These results emphasize that human monocyte-derived DC are highly efficient at cross-presentation, and demonstrate differences between monocyte-derived DC and monocytic lineage THP-1 cells.

Proteasome activity is required for cross-presentation induced by Ag-expressing E. coli

We hypothesized that both lysosomal and cytosolic proteases might be important for processing of Ags contained within *E. coli*. To test this possibility, two inhibitors of lysosomal proteases were used. Leupeptin and E64, two cysteine protease inhibitors with broad specificity, were added at different concentrations during the incubation of DCs with M1/LLO-expressing *E. coli*. After 6 h incubation, DCs were fixed with 0.5% PFA on ice and washed extensively before M_{58-66}-specific T cells were added into the ELISPOT plate. Neither leupeptin nor E64 (Fig. 6) inhibited the presentation of M_{58-66}, suggesting either that degradation in the phagolysosome was not required or that additional proteases are involved. Further studies using bafilomycin and chloroquine to block lysosomal acidification were next performed. Both drugs were able to inhibit presentation at concentrations typically used to inhibit Ag presentation (data not shown). However, we have observed significant toxicity and alterations in cell morphology induced by these drugs that is particularly pronounced for monocyte-derived DC. We therefore conclude that nonspecific alterations in DC function are likely to be responsible for the observed inhibition of Ag presentation function.

In contrast, a clear requirement for processing by the proteasome was observed. Proteasome inhibitors, clasto-lactacystin β-lactone, MG132, and PSI, were each able to block presentation when included during incubation of *E. coli* with DC before fixation. The presentation of M_{58-66} epitope by DCs was completely abolished with increasing concentration of all proteasome inhibitors as shown in Fig. 6. Proteasome inhibitors did not impair presentation of M_{58-66} peptide pulsed onto DC (our unpublished data).

Discussion

In this study we have demonstrated that recombinant *E. coli* expressing LLO is able to deliver an exogenous viral Ag to MHC class I-restricted Ag-processing pathway in a human in vitro system. The expression of LLO by *E. coli* is necessary for the Ag

FIGURE 4. Viability requirements for cross-presentation of M1 epitope by THP-1 cells analyzed by IFN-γ ELISPOT. *A, E. coli* treated with antibiotics gentamicin (100 μg/ml) or kanamycin (50 μg/ml) for 1 h were capable of stimulating cross-presentation following uptake by THP-1 cells. After treatment with each antibiotic, bacteria were plated and colonies counted to confirm that bacteria were killed. Reduction in viability of ~10^9-fold was observed. *B*, PFA fixation of *E. coli* decreased presentation of M1 epitope in a time-dependent manner. Bacteria were washed and resuspended in PBS before fixation as described in *Materials and Methods*.

FIGURE 6. Inhibitors of the proteasome, but not lysosomal hydrolases, blocked cross-presentation. DCs were fixed with PFA after a 5-h incubation with *E. coli* expressing M1 and LLO in the presence of different concentrations of leupeptin, E64, clasto-lactacystin β-lactone (lactacystin), PSI, or MG132. After fixation, DCs were washed and presentation analyzed by IFN-γ ELISPOT using M_{58-66}-specific T cells.

cross-presentation, as T cell responses were not detectable when using *E. coli* expressing M1 protein alone, even at ratios of bacteria to DC of 300:1. This is in contrast to published results in a mouse system using OVA as Ag, in which similarly high concentrations of bacteria expressing Ag without LLO were able to stimulate T cell hybridoma (14). This may reflect a difference between mouse and human APC, the individual Ag tested, or the sensitivity of primary T cells used in our study compared with the previously used T cell hybridoma. In both human and mouse systems, LLO-enhanced Ag delivery required cytosolic processing of Ag as indicated by sensitivity to proteasome inhibition.

Although THP-1 cells were able to cross-present Ag delivered by *E. coli* expressing LLO, DCs were shown to be more efficient based on the lower multiplicity of infection required to stimulate T cells. In fact, a decline in the stimulatory capacity of DC was noted at higher multiplicity of infection, possibly due to excessively strong stimulation of T cells, leading to anergy or cell death. Alternatively, higher concentrations of bacteria might be selectively toxic to DC. In addition, using Ag-pulsed fixed cells we showed that the T cell epitope was generated and presented at the surface of DC within 2–4 h, whereas THP-1 cells required 2–3-fold longer for maximal processing and presentation. We cannot currently explain this finding, but it may reflect inherent differences between DC and macrophage-like cells in Ag processing efficiency or bactericidal capacity. It will be necessary to closely follow the kinetics of Ag degradation and subsequent localization within each APC type to address this important question.

Because we failed to stimulate class I-restricted responses using *E. coli* expressing M1 Ag alone, we wanted to test whether opsonization of bacteria could enhance cross-presentation of expressed recombinant Ags. Other groups have shown that immune complexes taken up by DC can efficiently deliver Ags to the cytosol, whereas soluble Ags typically do not have such access (8, 9). Although opsonization clearly enhanced uptake of bacteria, presentation via the class I pathway still required coexpression of LLO.

The potential use of live bacterial vectors in vivo raises issues of safety, even with the use of *E. coli* such as BL21 that are considered nonpathogenic. We therefore evaluated the ability of bacteria killed by several different means to stimulate cross-presentation in our system. Both kanamycin and gentamicin were effective in killing the bacteria, while preserving their antigenic stimulatory capacity. This is similar to results previously reported in the mouse system (13). PFA fixation however diminished presentation of the M1 epitope, possibly due to susceptibility of LLO to cross-linking (D. Higgins, unpublished observations and Refs. 12, 13).

We have recently immunized HLA-DR1 transgenic mice with purified M1 protein to obtain a class II-restricted T cell hybridoma (P. Q. Hu, R. J. Tuma-Warrino, M. A. Bryan, K. G. Mitchell, D. E. Higgins, S. C. Watkins, R. D. Salter, and D. Canaday, manuscript in preparation). In addition to recognizing THP-1 cells or macrophages from HLA-DR1 donors pulsed with recombinant M1 protein, the hybridoma also recognizes influenza-infected cells, suggesting that it is specific for an epitope of M1 (our unpublished observations). We have shown that APC exposed to *E. coli* expressing either M1 alone or M1 plus LLO can stimulate the hybridoma (our unpublished observations). This demonstrates that both class I- and class II-restricted responses can be stimulated using LLO-containing *E. coli*, and that the processing pathways are not mutually exclusive. This is in contrast to results from the OVA system in mice showing that introduction of LLO into Ag-containing *E. coli* abolished their capacity to stimulate class II responses (14). This finding has implications for the use of bacterial vectors to induce immune responses in vivo, because both MHC class I- and class II-restricted responses could potentially be generated using a single construct.

Vaccination using bacterial vectors expressing subunit Ags has several distinct benefits that may be advantageous over other methods: they are inexpensive to manufacture on a large scale; a variety of recombinant proteins can be conveniently expressed and laborious

purification of these proteins is not required; common bacterial species are well studied and are easier to manipulate than viral vectors; bacteria contain compounds such as LPS that can stimulate APC via Toll-like receptors, activating APC appropriately during Ag uptake; and directed targeting to APC in vivo such as DC occurs normally and requires no further manipulation. Recombinant bacterial vaccine vectors have previously been tested in mouse models in vitro and in vivo. These vectors include bacille Calmette-Guérin (BCG) (27–29), *Listeria monocytogenes* (30–33), *Salmonellae* (34–38), *Shigellae* (39, 40), or *E. coli* expressing LLO (12, 14). Although immune responses against recombinant Ags were often generated in the mouse system, this does not always translate to humans. For example, vaccination with rBCG as Ag carrier produced Ag-specific Th1 response and protective serum Ab against *Plasmodium yoelii* in rodents (41). In a clinical trial involving human subjects, the intradermal administration of rBCG vector failed to stimulate Ab responses against delivered Ag (42). Nevertheless, studies of both in vitro and in vivo in mice suggest the potential value of these vectors, and raises the possibility of their eventual use in humans.

Acknowledgments

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