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Role of annexins in endocytosis of antigens in immature human dendritic cells

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SUMMARY

We have evaluated the uptake of a soluble protein antigen, denitrophenylated human serum albumin (DNP-HSA), and two different intracellular bacteria; *Chlamydia trachomatis* serovar L2 and *Mycobacterium tuberculosis* strain H37Ra, by immature human dendritic cells. These were generated by culturing progenitor cells from blood in the presence of cytokines (granulocyte–macrophage colony-stimulating factor and interleukin-4). Dendritic cells play a crucial part in antigen presentation for the induction of T-cell-dependent immune responses in various tissues. Recently, macropinocytic and phagocytic activity has been shown for immature dendritic cells of mouse, rat and human origin. In the present study, macropinocytosis characterized the uptake of the soluble protein-antigen DNP-HSA, whereas the *C. trachomatis* were ingested via receptor-mediated endocytosis in coated pits, and opsonized *M. tuberculosis* via phagocytosis. To follow the intracellular routes of the antigens, their positions were compared with the localization of annexins, a family of Ca^{2+}-and phospholipid-binding proteins, involved in membrane fusion, aggregation and transport of different vesicles. To elucidate further the intracellular pathway of the antigens, two other proteins, lysosome-associated membrane protein-1 (LAMP-1) and cathepsin D, were labelled. They are known to colocalize with major histocompatibility complex class II compartments in the immature dendritic cells. We observed a distinct translocation of annexin V to DNP-HSA containing endosomes, and annexin III to vesicles with *C. trachomatis*. Furthermore, annexin III, IV and V redistributed to phagosomes with *M. tuberculosis*. Both LAMP-1 and cathepsin D colocalized with DNP-HSA endosomes, and with phagosomes with *M. tuberculosis*. Thus, immature human dendritic cells have the capacity to phagocytose. Moreover, the handling of these antigens by dendritic cells may represent three distinct intracellular pathways, albeit some properties and compartments are shared.

INTRODUCTION

Dendritic cells (DC) play a crucial role in antigen-specific immune responses. they are potent, highly specialized antigen-presenting cells (APC).[1] The major role of DC is to acquire exogenous antigens in the peripheral tissues and present antigen peptides via major histocompatibility complex (MHC) class I and class II molecules to T lymphocytes in peripheral lymphoid organs.[2] The pathways for handling different kinds of antigens used by DC, as generated by culturing progenitor cells from blood with granulocyte–macrophage colony-stimulating factor (GM-CSF) and interleukin-4 (IL-4), are thus of interest since they should mimic functions of *in vivo* immature (interstitial) DC found in non-lymphatic tissues.

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The antigen presentation is very effective in DC, and therefore even a low capacity of antigen uptake is sufficient. Indeed, only a few hundred peptide molecules need to be presented to accomplish good stimulation of primary T cells by the mature DC.[3] The role of DC as APC *in situ* has been demonstrated for several types of antigens, e.g. transplantation antigen,[4] foreign protein,[2] and influenza A proteins.[5] The migration of rat DC from non-lymphoid tissues to lymphoid organs after antigen challenge has also been demonstrated *in vivo*.[6] Several studies support the proposition that DC are phagocytic at immature stages of their life cycle. Phagocytic activity has been shown *in vitro* for freshly isolated Langerhans' cells (LC), and *in vivo* for proliferating DC precursors from mouse bone marrow as well as for immature rat DC.[6–8] There are two identified distinct ways for antigen capture by immature DC, via macropinocytosis (MP) and lectino-endocytosis via mannose receptors (MR).[9] Human immature DC uptake and processing of larger particulate antigens such as bacteria, are however, not well characterized.

Larsson *et al.*[10] recently showed that mature human DC

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express annexin I, III, IV, V and VI. Other studies have suggested that annexins are useful correlates of intracellular antigen pathways.[11,12] The annexins are involved in vesicle aggregation, movement and membrane fusion, and may mediate Ca^{2+}-regulated traffic during endo- and exocytosis.[13]

To elucidate further the pathways and fates of different kinds of antigens we followed the endocytosis of dinitrophenylated human serum albumin (DNP-HSA), *Chlamydia trachomatis* serovar L2 and *Mycobacterium tuberculosis* strain H37Ra in our immature DC. In parallel, the localization of annexin I, III, IV, V and VI, lysosome-associated membrane protein-1 (LAMP-1) and cathepsin D was determined immunofluorescently using confocal microscopy. Our data suggest that the three antigens were handled distinctly; DNP-HSA was initially found in macropinosomes and was later mostly exocytosed. Annexin V, LAMP-1 and cathepsin D were translocated to vesicles with DNP-HSA. *Chlamydia trachomatis* apparently entered cells via receptor-mediated endocytosis in coated pits and was found in individual endosomes. After about 5 hr inside the DC *C. trachomatis* elementary bodies (EB) were exocytosed. Annexin III translocated specifically to vesicles with *C. trachomatis* EB. *Mycobacterium tuberculosis* had to be opsonized with human serum before efficient phagocytosis occurred. The bacteria were retained in phagosomes in the DC and colocalized with the marker proteins, annexin III, IV and V, LAMP-1 and cathepsin D. Immature DC have thus the ability to phagocytose bacteria. The fates of these three antigens, DNP-HSA, *C. trachomatis* and *M. tuberculosis* in DC may represent three intracellular routes sharing some properties and compartments.

MATERIAL AND METHODS

Culture medium

RPMI-1640 (Gibco Laboratories, Grand Island, NY) was supplemented with 20 μg/ml gentamicin, 200 mM glutamine, 50 μM 2-mercaptoethanol, 10% heat inactivated fetal calf serum (FCS), 10 mM HEPES buffer, recombinant human cytokines, 1000 U/ml GM-CSF (Schering-Plough/Sandoz, Bern, Switzerland), and 700 U/ml IL-4 (Genzyme Corp. Cambridge, MA).

Culture of human dendritic cells

The method of Romani *et al.*[14] was used with slight modification. Buffy coats were used to isolate mononuclear cells, while separation was done on a density gradient of Ficoll–Hypaque (1·077 g/ml, Pharmacia, Uppsala, Sweden) which was followed by the depletion of T cells by rosetting with neuraminidase-treated sheep red blood cells. The cells obtained were cultured in a concentration of $0{\cdot}5 \times 10^6$–$0{\cdot}7 \times 10^6$ cells/ml in the culture medium supplemented with GM-CSF and IL-4. These DC were used from day 8 to day 10 of culture.

Characterization of human DC by flow cytometry

The phenotype of the DC was analysed using an EPICS Profile I flow cytometer with Power Pac (Coulter Electronics Corporation, Hialeah, FL). DC were stained with human-specific mouse monoclonal antibodies (mAb; Fig. 1) followed by diluted 1:100 rabbit anti-mouse $F(ab')_2$ fragments conjugated with fluorescein isothiocyanate (FITC) (Dakopatts, Copenhagen, Denmark). Antibodies were obtained from Dakopatts, Serotec (Oxford, UK) or Becton Dickinson (Stockholm, Sweden). Mouse mAb used were anti-HLA DR, CD1a, CD4, CD11a, CD11b, CD11c, CD18, CD25, CD29, CD31, CD32, CD41, CD43, CD44, CD54, CD58 and the cocktail contained anti-CD3, CD14, CD19 and CD56.

Cell permeabilization

DC (100 000/well) were washed twice in phosphate-buffered saline (PBS) with 1% FCS, 1% human serum (HS) and 0·2% sodium azide (washing buffer, WB) by spinning the cells at 480 ***g*** for 2 min. Then the cells were fixed with 4% paraformaldehyde in PBS (pH 7·3) for 10 min on ice, washed twice and permeabilized with 1% saponin (Sigma) in washing buffer for 30 min on ice. The DC were washed twice in WB and then resuspended in 100 μl WB with 0·1% saponin.

Staining of non-permeabilized or permeabilized DC for flow cytometry

The fixed and permeabilized DC suspensions were incubated with 30 μl of either polyclonal rabbit-anti-human antibodies against annexin I, III, IV, V, or VI or the mouse-anti-human mAb against HLA DR and CD8 for 30 min on ice and washed twice in the presence of 0·1% saponin. Staining of HLA DR was used as a positive control and CD8 as a negative control. Cell pellets were resuspended in 100 μl of WB with 0·1% saponin and 30 μl of FITC-conjugated swine anti-rabbit $F(ab')_2$ fragments (Dakopatts; 1:200) for the annexin stained DC and 30 μl mouse anti-rabbit $F(ab')_2$ 1:200 for the HLA DR- and CD8-stained DC and were further incubated for 30 min on ice, and then washed as above. Unbound antibodies were quenched with 30 μl rabbit immunoglobulin-fraction (1:100) or mouse serum (1:100) and 100 μl of WB with 0·1% saponin for 30 min on ice. The cells were then washed once in WB with 0·1% saponin and twice in buffer without saponin. The unpermeabilized and fixed DC were prepared similarly but without the WB.

Endocytosis of DNP-HSA by human DC

Dendritic cells were incubated for 1, 2, 4, 10, 20, 30, or 60 min at 37°, with 0·66 mg/ml DNP-HSA (DNP/HSA molecular ratio 9·8) conjugated with FITC (FITC/HSA molecular ratio 9·8) for visualization. Endocytosis was stopped at each time-point with ice-cold RPMI-1640. The cells were washed three times by centrifugation for 5 min at 100 ***g*** at 4° and then chased at 37° for 0, 10, 30, or 60 min to allow further endocytosis and exocytosis. Cytocentrifuge preparations were made at all the chase times. The endocytosis of DNP-HSA by DC was performed >10 times.

Infection of DC with C. trachomatis *serovar L2*

Stock of *C. trachomatis* serovar L2 was harvested from McCoy cells as described elsewhere.[15] DC were washed and then resuspended three times in RPMI-1640 without serum. *Chlamydia trachomatis* was added to the cell suspension at a multiplicity of infection of 500 EB/DC (4×10^6–5×10^6) and inoculated for 2 hr at 37°. The cell suspension was gently shaken to give an even distribution of EB. After inoculation with the bacteria, cells were washed twice in RPMI-1640 to remove unbound bacteria and then reincubated for 0·5, 1, 2, 4, 6, 8, 12, 16, or 24 hr in culture medium. Infection of DC with *C. trachomatis* serovar L2 was performed <10 times.

Infection of DC with M. tuberculosis *strain H37Ra*

The strain H37Ra (American Type Culture Collection 25 177) of *M. tuberculosis* was used in the log phase of growth. The avirulent *M. tuberculosis* strain H37Ra was transformed with the plasmid, pFPV-2, as described by Valdivia *et al.*[16] The bacteria were centrifuged and resuspended in PBS buffer (pH 7·2) and separated to a single bacterium suspension by the suction force of syringe several times. Then most of the bacteria (85–95%) were single as determined microscopically. DC were washed three times in RPMI medium without serum and then resuspended in RPMI culture medium. The Mycobacteria were opsonized with 50% fresh human serum (HS) in PBS for 30 min at 37° and washed twice in RPMI. Bacteria and cells were then incubated at a bacteria to cell ratio 40:1 (40 bacteria/DC, a final cell concentration of 5×10^6 DC/ml) for 0·25, 0·5, 1, 3, 6, 9, 12, 24 and 48 hr at 37°. Samples were taken at the indicated intervals, washed twice in RPMI-1640 and spun down on glass slides. Infection of DC with *M. tuberculosis* was performed seven times.

Staining of annexins and Chlamydia proteins in antigen containing DC

Cytospin preparations were made in a cytocentrifuge (Shandon Southern Instruments Inc. Sewickley PA) by loading 20×10^4–30×10^4 cells/slide. Slides were then stored with desiccant at $-20°$ until use. The slides were fixed in acetone for 10 min in room temperature (RT). Slides with fixed *C. trachomatis*-infected DC were preincubated with normal donkey serum 1:25 (Jackson Immunoresearch, West Grove, PA), followed by mouse anti-major outer membrane protein (MOMP) monoclonal antibody (1:2000) (Washington Research Foundation, Seattle, WA) for 45 min at 37°. The cytospins were then washed 3×3 min in PBS containing 0·5% bovine serum albumin (BSA) and 0·5% milk powder (PBS/BSA/M) and incubated with 1:100 dilution of FITC-conjugated donkey anti-mouse $F(ab')_2$ fragments (Jackson Immunoresearch) for 45 min. Slides with fixed DC infected with *M. tuberculosis*, with endocytosed DNP-HSA, or infected with *C. trachomatis* (MOMP stained) were washed 3×3 min in PBS/BSA/M and incubated for 30 min with normal swine serum (1:20, NSS) (Dakopatts) at RT in a moist chamber. The excess of NSS was removed, and the cells were overlaid with rabbit polyclonal antibodies, raised against purified human annexin proteins for 45 min at 37°. Anti-annexin I and V antibodies were diluted 1:150, anti-annexin III and IV antibodies were diluted 1:40, and anti-annexin VI antibodies were diluted 1:25 in PBS/BSA/M. The cells were washed 3×3 min in PBS/BSA/M. Rhodamine-conjugated swine anti-rabbit IgG (Dakopatts) diluted 1:200 in PBS/BSA/M was added, and the cells were further incubated for 45 min at 37° and thereafter rinsed as above.

LAMP-1 and cathepsin D staining in DC incubated with M. tuberculosis *or DNP-HSA*

Slides with infected DC were fixed in acetone for 10 min and washed three times in PBS/BSA/M and then incubated with normal rabbit serum (1:25; NRS) (Dakopatts) for 30 min at RT. The excess of NRS was removed and the slides were incubated with anti-cathepsin D (1:100; Dakopatts) or LAMP-1 (1·50) antibodies for 45 min at 37° in a moist chamber and washed 3×3 min in PBS/BSA/M. Incubation with rhodamine-conjugated rabbit anti-mouse antibody (1:100; Dakopatts) for 45 min at 37° was followed by 3×3 min washing.

Confocal and fluorescence microscopy

The slides with DC containing DNP-HSA, *M. tuberculosis,* or *C. trachomatis* were examined in a confocal laser scanning microscope (Sarastro 2000; Molecular Dynamics, Sunnyvale, CA) equipped with a $\times 60$ lens (numerical aperture = 1·4).[11] The cells were serially scanned in horizontal sections 0·2 μm apart. To excite both fluorescein and rhodamine the 514 nm line of the Sarastro 2000 was used. The dicroic beam splitter (LP530) and barrier filters in front of the detector 1 (570EFPL) and detector 2 (540DF30) allowed separation of the fluorescent markers. With the standard fluorescence microscope the excitation and emission wavelengths were 450–490 nm and 520 nm for fluorescein, and 546 nm and 590 nm rhodamine, respectively.

RESULTS

Phenotype and functional characterization of the dendritic cells

Typical processes and veils were seen on cells at the rim of the cell aggregates in the DC cultures. Some of these cells were also released into the medium. Good allogenic stimulation by DC in mixed lymphocyte reaction was found in all cultures and was performed as a control of the stimulatory function (data not shown). Analysis of the cell phenotype for expression of typical proteins revealed that our cultures displayed MHC class II and adhesion molecules with the same patterns as the DC cultured by Romani *et al.*[14] and Sallusto *et al.*[17] The cells were negative for the antibodies, anti-CD3, CD14, CD19 and CD56, used to stain markers for T cells, macrophages, B cells and natural killer (NK) cells, respectively (Fig. 1).

Characterization of annexin expression

To determine whether annexin I, III, IV, V and VI were expressed at the plasma membrane and/or intracellularly, we analysed normal and permeabilized DC with flow cytometry. All annexins were weakly expressed on the surface and strongly expressed intracellularly (Fig. 2). The distribution of annexin I, III, IV, V, or VI along the plasma membranes in the DC population was heterogeneous (Fig. 2).

Endocytosis of DNP-HSA and colocalization with annexins in DC

After only 2 min of incubation of the soluble DNP-HSA, vesicles were seen in the DC in sizes estimated as macropinosomes, i.e. 0·5–3 mm (Fig. 3). After 20 min of incubation with DNP-HSA, all DC contained numerous vesicles, but the number and size differed between cells. The DNP-HSA vesicles became more aggregated after the chase period and moved towards the perinuclear area. After 20-min incubation and 60-min chase, fewer vesicles were seen and more closer to the plasma membrane, probably due to the exocytosis of previously ingested DNP-HSA. Annexin V was colocalized to a large extent with the endocytosed DNP-HSA. This was seen almost directly upon uptake of DNP-HSA (Fig. 4). None of

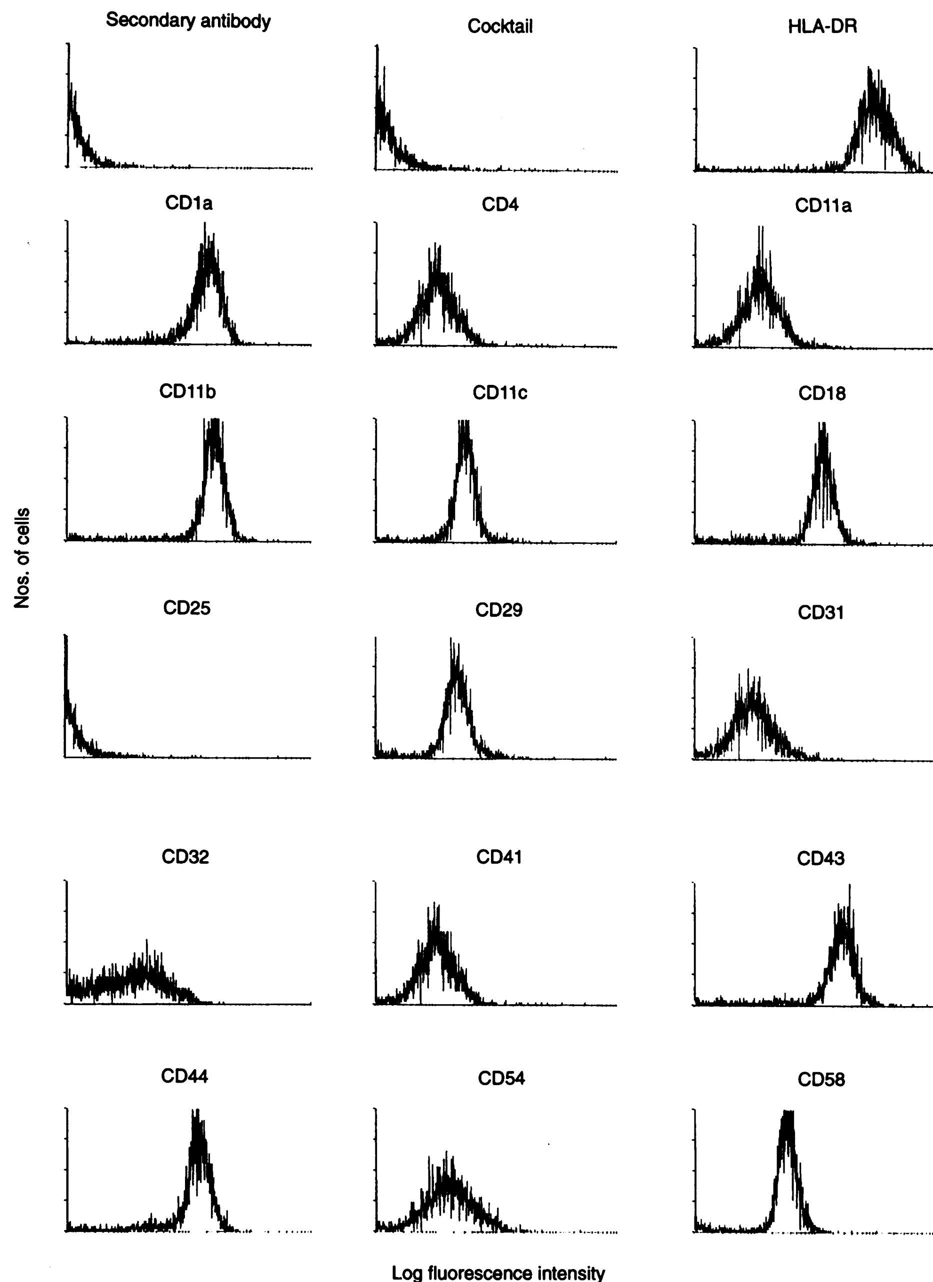

Figure 1. Antigen histograms of immature human DC generated from cytokine (GM-CSF and IL-4) culture of progenitor cells from blood monitored by flow cytometry. The phenotypic analysis of the DC was done by staining with indicated mouse mAb followed by FITC-conjugated rabbit anti-mouse $F(ab')_2$ fragments. A representative experiment is shown. The staining cocktail contained anti-CD3, -CD14, -CD19 and -CD56 antibodies and was used to visualize contaminating cell types such as T cells, B cells, macrophages and NK cells, respectively.

the other annexins, i.e. I, III, IV and VI, colocalized with endocytosed DNP-HSA in DC.

C. trachomatis infection and localization of annexins

After 2 hr of incubation with *C. trachomatis*, most of the DC had taken up bacteria with different efficiency. Some cells contained many vesicles with *C. trachomatis*. The vesicles were initially found near the plasma membrane, but as the infection progressed, i.e. after 3–4 hr, the vesicles moved towards the perinuclear area. After 5–8 hr, exocytosis was evident and thereafter, fewer and fewer bacteria were detected inside the DC. After 24 hr some cells had re-endocytosed previously released *C. trachomatis*. The *C. trachomatis* was in the infectious elementary body (EB)-phase and did not enter into the reticular body (RB)-phase of the well-established biphasic life cycle. Only around 1% of the DC had large typical inclusions of fused vesicles containing EB. Annexin III was the only

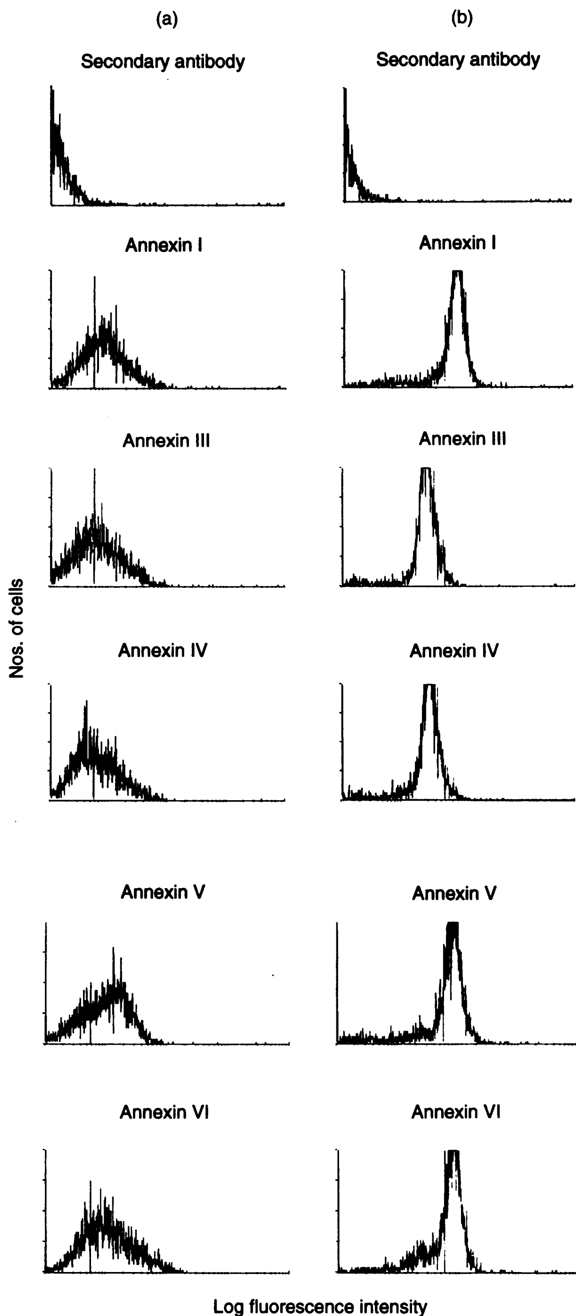

Figure 2. The extracellular surface exposure (a) and total expression (b) of annexins by human immature DC from a representative experiment. (a) The DC were fixed and then stained with anti-annexin I, III, IV, V and VI antibodies, followed by rabbit anti-mouse $F(ab')_2$ fragments; (b) the DC were fixed, permeabilized by saponin and then stained with anti-annexin I, III, IV, V and VI antibodies followed, by rabbit anti-mouse $F(ab')_2$ fragments.

annexin examined that colocalized with vesicles containing *C. trachomatis* (Fig. 5). This occurred, however, first after 2 hr of infection. It then increased with time.

M. tuberculosis infection and distribution of annexins

There were very few opsonized *M. tuberculosis* bacteria seen in the DC after 15 min of infection, but the proportion of infected DC increased during the incubation time; at 1 hr approximately 40%, at 3 hr 80% and at 24 hr more than 90% of the cells were infected. The infected DC contained one to four bacteria per cell after 1 hr of infection. Some *M. tuberculosis* aggregates were observed in the infected cells after 1 hr but decreased with time. After 24 hr of infection, the DC viability was around 95% and the total number of cells had decreased by 10%. The DC seemed to become activated by the bacterial interaction, since more and longer dendrites and more veils were seen after 24 hr. The bacteria were seemingly retained intracellularly in phagosomes and were not exocytosed to any appreciable extent. When investigating in parallel the position of annexins and *M. tuberculosis* in DC, annexin I and VI did not colocalize with bacteria, whereas annexins III, IV and V all translocated to the phagosomes containing bacteria (Fig. 6). Annexin III translocation was detected after only 15 min of infection and increased with time. There was also a tendency towards colocalization between annexin IV and V and *M. tuberculosis* after 15 min, although to a smaller extent than for annexin III. However, after 24 hr of infection, the translocation of annexin IV and V to phagosomes was even higher than for annexin III.

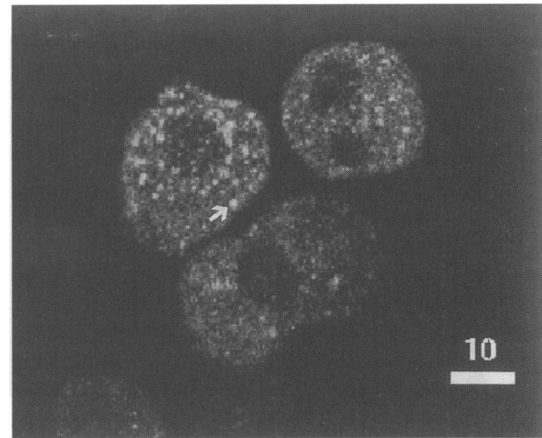

Figure 3. Macropinocytosis in human immature DC generated by culturing progenitor cells from blood with cytokines (GM-CSF and IL-4) after incubation for 2 min with DNP-HSA. Arrow indicating a vesicle of the size 1·5–2·0 µm. All DC contained numerous vesicles with DNP-HSA, but the number and size differed between cells. The bar represents 10 µm.

LAMP-1 and cathepsin D colocalization with *M. tuberculosis* and DNP-HSA

Immunofluorescence staining revealed that LAMP-1-containing vesicles were abundant in the DC, whereas cathepsin D displayed a homogeneous cytoplasmic rather than a vesicular distribution. LAMP-1 staining was moderate in some DC and strong in others. Colocalization between LAMP-1- and DNP-HSA-containing vesicles was visible after 20-min incubation and 30-min chase and was only localized near the nucleus in a few vesicles (Fig. 7a–c). This was also seen for cathepsin D and vesicles with endocytosed DNP-HSA after 20 min and it increased with time (Fig. 7 d–f). Cathepsin D appeared together with DNP-HSA to a higher degree than LAMP-1. In the cells infected with *M. tuberculosis*, there was

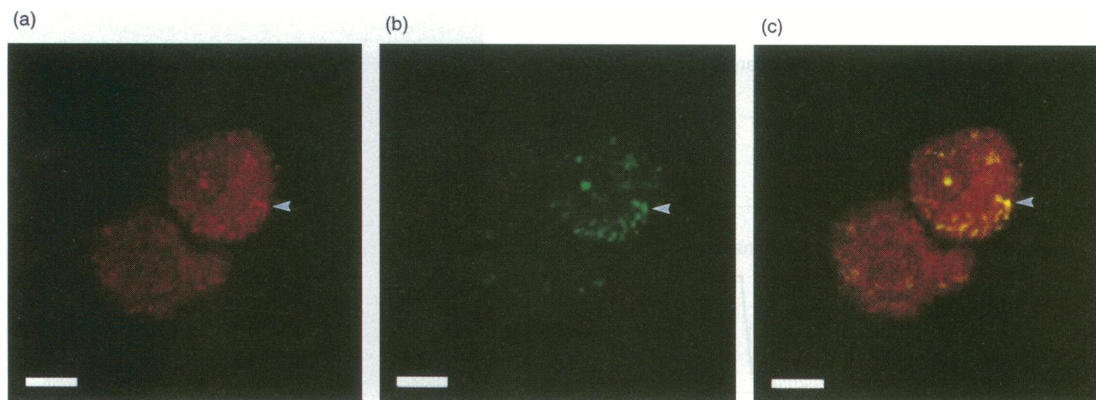

Figure 4. Confocal image sections of immature human DC showing vesicles with endocytosed DNP-HSA after 20-min of incubation and 10-min chase and the pattern of annexin V expression: (a) DC with endocytosed DNP-HSA stained with annexin V antibody followed by rhodamine-conjugated swine anti-rabbit antibody; (b) DC with DNP-HSA vesicles; (c) anaglyph image, produced by combined (a) and (b) images; The images a–c show the same cells and from the same section. The arrowhead in the DC indicates translocation of annexin V to vesicles containing DNP-HSA. Depending on their position in the endocytotic pathway, not all vesicles containing DNP-HSA were decorated with annexin V. The bar represents 10 μm. The images are showing representative DC.

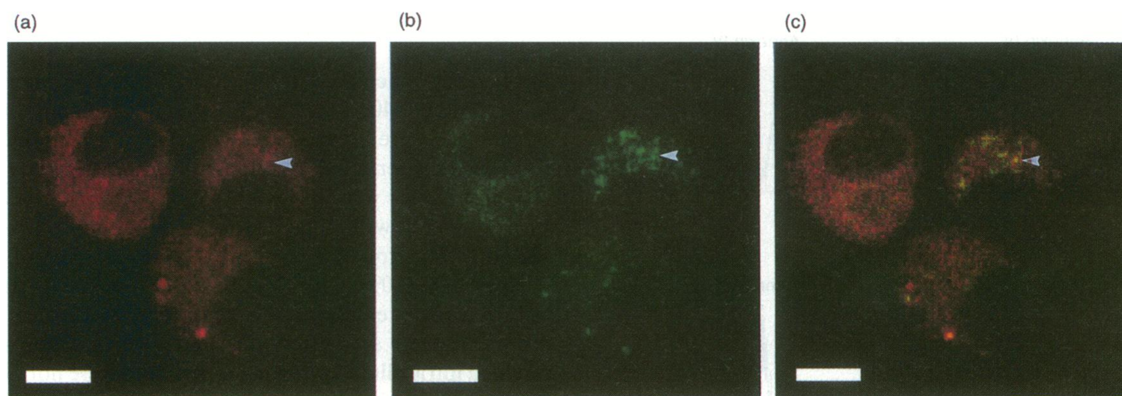

Figure 5. Confocal image sections showing immature human DC infected 2 hr with *C. trachomatis* serovar L2 and chased for 0·5 hr, and the distribution of annexin III: (a) DC infected with *C. trachomatis* and stained with annexin III antibody followed by rhodamine-conjugated swine anti-rabbit antibody; (b) DC infected for 2 hr with *C. trachomatis* elementary bodies and chased 0·5 hr. (c) Anaglyph image, produced by combined (a) and (b) images; Vesicles containing elementary bodies were visualized with antibodies to *C. trachomatis* membrane protein (MOMP) followed by FITC-conjugated donkey anti-mouse $F(ab')_2$ fragments. The arrowhead indicates translocation of annexin III to vesicles containing *C. trachomatis* elementary bodies in the DC. The bar represents 10 μm. The images are showing representative DC.

colabelling of phagosomes and LAMP-1 (Fig. 8a–c) and cathepsin D (Fig. 8d–f) at an early phase of infection. In contrast to DNP-HSA, LAMP-1 colocalized with *M. tuberculosis* to a higher extent than did cathepsin D.

DISCUSSION

Dendritic cells (DC) generated from progenitor cells in blood appear to resemble interstitial DC. These are thought to be an immature end-stage for DC recently produced by terminal division of precursor cells originating from bone marrow.[6] Freshly isolated Langerhans' cells (LC) and human immature DC express the Fcγ receptors (FcγR), complement receptors (CR) and mannose receptors (MR), which should help them to bind and target the antigens to the antigen-presenting machinery.[9,18,19] During maturation of freshly isolated DC, i.e. culturing of LC and blood DC, both FcR and MR expressions are down-regulated together with the capacity to process antigens, whereas the expression of costimulatory molecules CD40, CD80 and CD86 is increased.[9,18] Addition of lipopolysaccharide (LPS) and tumour necrosis factor-α (TNF-α) accomplish similar phenotypic changes in immature DC.[9]

There are at least three possible ways for the uptake of soluble proteins in DC, i.e. via coated pits, macropinocytosis, or Birbeck granule (found only in LC).[18] In the immature stage *in vivo*, the DC display transient phagocytic activity on particulates, a necessary feature for the induction of immune responses against bacteria, such as *M. tuberculosis*.[6] The soluble protein, DNP-HSA, most likely follows the endocytic pathway(s) for soluble proteins in immature DC, whereas large microbial antigens such as *M. tuberculosis* are phagocytosed, e.g. via the receptor-mediated uptake of large particles. Although phagocytosis and pinocytosis are two distinct mechanisms, they may partly involve the same compartments and molecules.[20] For instance in immature human DC, soluble

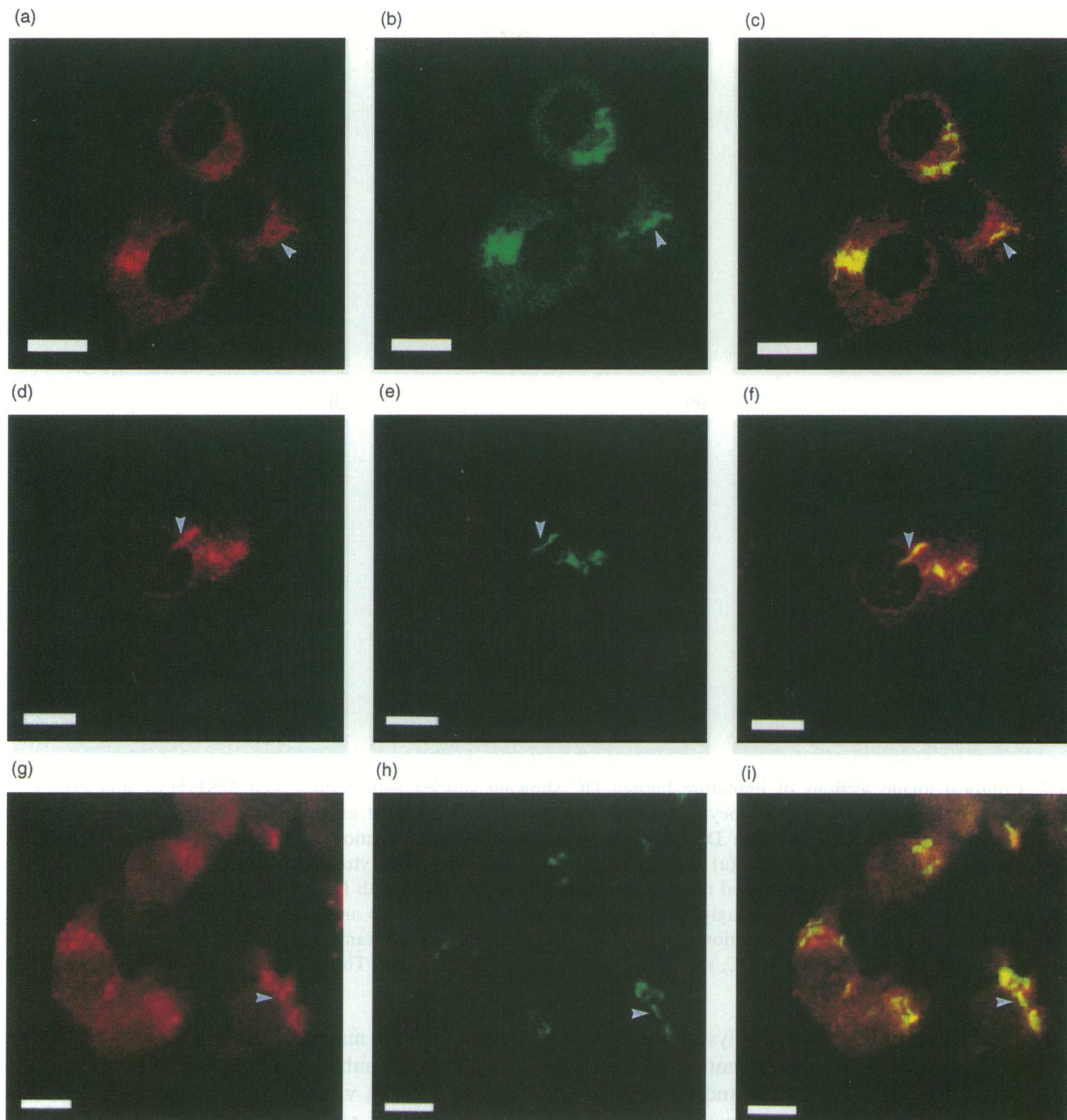

Figure 6. Confocal images of human DC infected with serum-opsonized avirulent *M. tuberculosis* and the localization of annexin III, IV, or V: (a) The *M. tuberculosis*-infected DC were stained with annexin III antibody followed by rhodamine-conjugated swine anti-rabbit antibody; (b) DC infected with *M. tuberculosis* for 6 hr; (c) anaglyph image, produced by combined (a) and (b) images; (d) *M. tuberculosis*-infected DC stained with annexin IV antibody followed by rhodamine-conjugated swine anti-rabbit antibody; (e) DC infected with *M. tuberculosis* for 6 hr; (f) anaglyph image, produced by combined (d) and (e) images; (g) The *M. tuberculosis*-infected DC stained with annexin V antibody followed by rhodamine-conjugated swine anti-rabbit antibody; (h) DC infected with *M. tuberculosis* for 24 hr; (i) anaglyph image, produced by combined (g) and (h) images. The (a–c), (d–f), or (g–i) images show the same cells and from the same section. Arrowheads in the images show translocation of annexin III (a–c), IV (d–f), or V (g–i) to phagosomes with opsonized *M. tuberculosis* in the DC, respectively. The bar represents 10 μm. The images are showing representative DC.

proteins and microbial antigens end up in the major histocompatibility class II compartment (MIIC).[9,21] Macropinocytosis allows continuous internalization of large volumes of fluid in immature DC. Our data support that macropinocytosis is used to internalize the soluble protein, DNP-HSA, by immature DC (Fig. 2). Receptor-specified endocytosis in immature DC is dependent on membrane-ruffling and actin rearrangement, which is also Ca^{2+}-dependent.[9] The selective mobilization of annexin V points to its putative function as a Ca^{2+} pore,[22] which should allow a Ca^{2+} increase needed for rearrangement of the actin cytoskeleton in close proximity to the endocytic vesicles.

The uptake of *C. trachomatis* by mammalian cells can be accomplished by receptor-mediated endocytosis via receptors bound to heparan sulphate-like ligands on the surface of the bacteria. This process takes place in coated pits and involves clathrin and actin.[15,23,24] Normally, the elementary bodies (EB) multiply intracellularly where they develop typical

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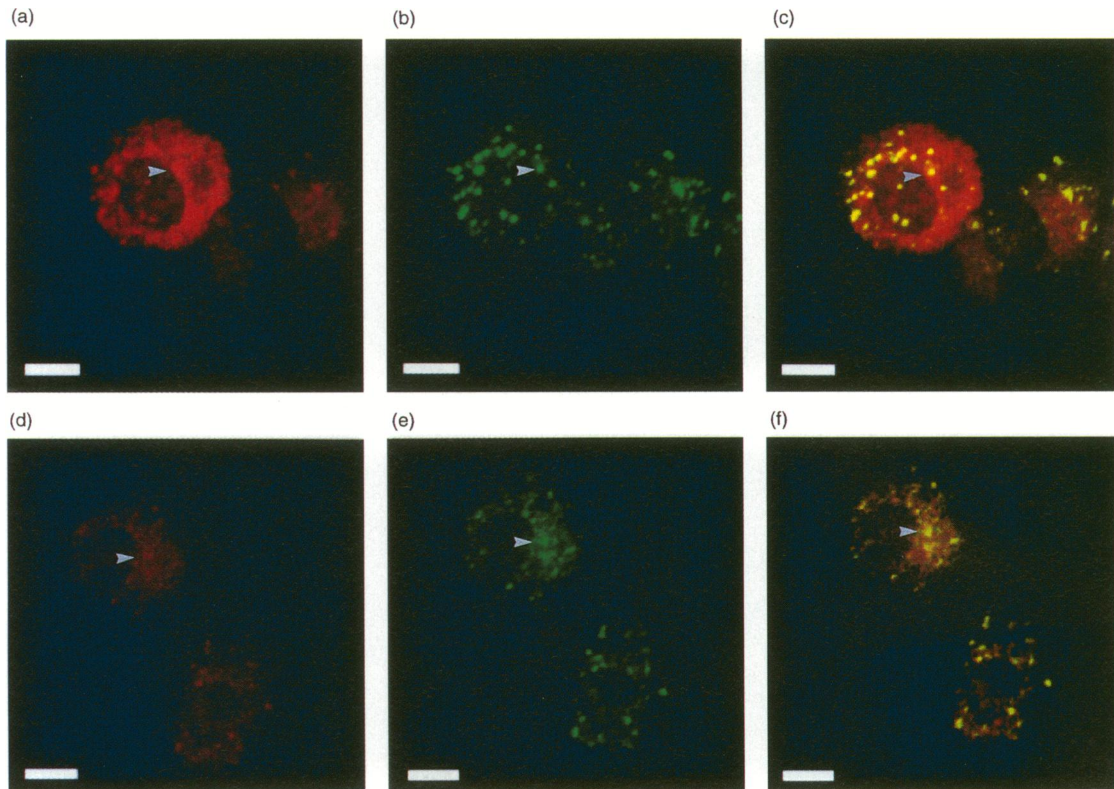

Figure 7. Confocal image sections of immature human DC showing vesicles with endocytosed DNP-HSA and expression of LAMP-1 or cathepsin D; (a) DC with endocytosed DNP-HSA stained with LAMP-1 antibody followed by rhodamine-conjugated rabbit anti-mouse antibody; (b) DC with DNP-HSA vesicles after 20-min incubation with DNP-HSA and 60-min chase; (c) anaglyph image, produced by combined (a) and (b) images; (d) DC with endocytosed DNP-HSA stained with cathepsin D antibody followed by rhodamine-conjugated rabbit anti-mouse antibody; (e) DC with DNP-HSA vesicles after 20-min incubation with DNP-HSA and 60-min chase; (f) anaglyph image, produced by combined (d) and (e) images; The (a–c) and (d–f) images show the same cells and from the same section. Arrowheads in the images indicate translocation of LAMP-1 (a–c) or cathepsin D (d–f) to vesicles with DNP-HSA in the DC, respectively. The bar represents 10 μm. The images are showing representative DC.

inclusions, and escape fusion with phagolysosomes.[25] The uptake of *C. trachomatis* by DC in the present study was not productive, since the EB did not multiply and develop large inclusions. Intracellular *C. trachomatis* were instead exocytosed from the DC after some time and were able to reinfect cells. In the DC the EB, thus probably avoided the lysosomal compartments but could still traffic through the MIIC, because mature DC cultured from blood are able to present and give Chlamydia-specific primary T-cell responses, both from antigen peptides and whole bacteria.[26] Intracellular Ca^{2+}-depletion in McCoy cells and HeLa cells inhibits aggregation of Chlamydial vesicles and translocation of annexin III to the large inclusions.[11] In fact, the infection of DC with *C. trachomatis* resembled that found in the Ca^{2+}-free environment in McCoy cells and HeLa cells, i.e. endosomes containing individual EB, but no large inclusions in the cytoplasm.[11]

An *in vivo* study on rat has indicated that the interstitial immature DC have phagocytic activity, but maturation down-regulates this function.[6] Phagocytosis in immature DC is slower than in macrophages, and DC are furthermore unable to engulf large objects as opsonized erythrocytes.[7,8] The phagocytosis of the present strain of *M. tuberculosis* by DC required opsonization with fresh human serum (HS), since very few DC ingested unopsonized bacteria. This has previously been found also for macrophages and neutrophils[27] (Majeed *et al.* submitted for publication), where it was proposed that phagocytosis of both virulent and avirulent strains was dependent on complement receptors, whereas the mannose receptors play an important role particularly in the ingestion of virulent strains.[28] Therefore, it is likely that the phagocytosis of opsonized *M. tuberculosis* by DC in this study involves the complement receptors, i.e. CR3 (CD11b/CD18) and CR4 (CD11c/CD18). These are expressed at high levels on the immature DC (Fig. 1). Proliferating immature mouse DC are better APC of mycobacterial antigens (Bacillus Calmette–Guérin) than cultured mature mouse DC.[8] Some processing of surface proteins of the Mycobacteria probably takes place in both our immature DC and the immature mouse DC, which is sufficient for presentation.[8]

The Ca^{2+}-dependent binding of annexins to phospholipid membranes may play many specific roles, including vesicle movement, aggregation and fusion, ion movement across membranes, signal transduction and interaction with cytoskeletal elements.[29,30] Annexins also participate in the movements of vesicles in eukaryotic cells, needed to carry out the handling and presentation of different antigens.[31] The expression of annexin I, III, IV, V and VI were higher in the immature and more endocytically active DC compared to mature DC.[10] We

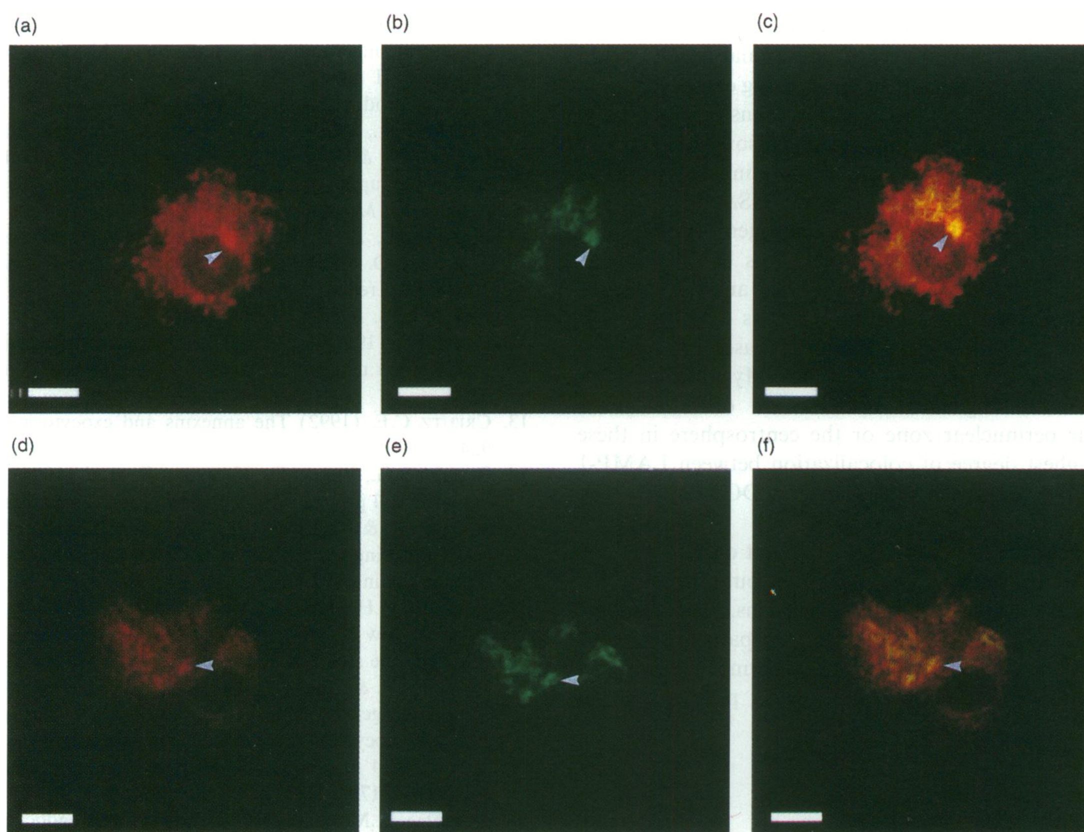

Figure 8. Confocal images of immature human DC infected with opsonized avirulent *M. tuberculosis* and the expression of LAMP-1 or cathepsin D: (a) the *M. tuberculosis*-infected DC were stained with LAMP-1 antibody followed by rhodamine-conjugated rabbit anti-mouse antibody; (b) DC infected with *M. tuberculosis* for 6 hr; (c) anaglyph image, produced by combined (a) and (b) images; (d) The *M. tuberculosis*-infected DC were stained with cathepsin D antibody followed by rhodamine-conjugated rabbit anti-mouse antibody; (e) DC infected with *M. tuberculosis* for 12 hr; (f) anaglyph image, produced by combined (d) and (e) images. The (a–c) and (d–e) images show the same cells and from the same section. Arrowheads in the images show translocation of LAMP-1 (a–c) and cathepsin D (d–f) to phagosomes with opsonized *M. tuberculosis* in the DC, respectively. The bar represents 10 μm. The images are showing representative DC.

found annexins at the plasma membrane of DC, which could indicate that they take this position for instance when endosomes are formed or leave via exocytosis. Annexins I and II have primarily been seen in association with early endosomes, whereas annexins IV and VI were more widely distributed. Annexin IV has been observed both with early and late endosomes.[10,20,31] Furthermore, during endocytosis and exocytosis of DNP-HSA in mature DC annexin V translocated to vesicles containing DNP-HSA.[10] The same was found for our immature DC (Fig. 4). We propose that annexin V specifically acts as a Ca^{2+} regulator at the plasma membrane and at vesicle membranes.

The association of annexins to early and late endosomes differs between cell types. We found that annexin III, IV and V translocated to phagosomes with *M. tuberculosis* in immature DC. Similar translocation of annexin III and IV was also observed in neutrophils, whereas annexin V was not affected in these cells (Majeed *et al.*, submitted for publication). The annexins III, IV and V associate with *C. trachomatis* EB-containing vesicles in McCoy cells and HeLa cells. By contrast, only annexin III colocalized with individual endosomes containing EB in immature DC (Fig. 5). Annexin IV was found in close proximity to *M. tuberculosis* in the DC. Phagosomes have been proposed to fuse with early endosomes and late endosomes during endocytosis, i.e. where annexin IV is located.[20] Annexin V was translocated to both endosomes with DNP-HSA and phagosomes with *M. tuberculosis*. It is therefore possible that annexin V associates with vesicles capable of fusing with early and late endosomes, and that also phagosomes fuse with such vesicles, since annexin V decorates both vesicles with DNP-HSA and phagosomes with *M. tuberculosis*.

Human monocyte phagosomes with virulent *M. tuberculosis* exhibit MHC class I and MHC class II molecules and endosomal markers, e.g. the transferrin receptor, LAMP-1, LAMP-2, CD63 and the lysosomal acid protease cathepsin D.[32] The *M. tuberculosis* phagosomes in these cells do not fuse with lysosomes, instead they retain endosomal characteristics and become mildly acidified. *Mycobacterium tuberculosis* bacteria resist destruction inside mononuclear phagocytes.[32] Immature DC differ from macrophages and polymorphonuclear phagocytes in that antigens are not totally degraded in lysosomes. Instead, they are partly degraded to peptides in a late endosome-like structure, the MIIC. Here MHC class II molecules, HLA DM, CD63, LAMP-1 and cathepsin D are found in human DC.[9,18,21] The amounts of LAMP-1 seen in

MIIC in mature DC are smaller than in immature DC.[9,33] The differences between mature DC and immature DC could depend on a decrease in the antigen-processing capacity during maturation. Our data show that there is translocation in the DC of LAMP-1 and cathepsin D to phagosomes containing *M. tuberculosis* and endosomes containing DNP-HSA. Cathepsin D has the capacity to degrade HSA in peritoneal macrophages and veiled DC during antigen processing.[34] Cathepsin D, an aspartyl protease may thus be one enzyme responsible for the processing of DNP-HSA and may also be responsible for processing of surface proteins on *M. tuberculosis* in our DC. DC progenitor from mouse have indeed, proved to be the most efficient APC for Mycobacteria and phagosomes containing these bacteria are often localized within a clear perinuclear zone or the centrosphere in these cells.[8] The highest degree of colocalization between LAMP-1 or cathepsin D with *M. tuberculosis* in our DC was also seen in this area.

To summarize, immature DC are able not only to endocytose free antigenic material like DNP-HSA, but also phagocytose potentially pathogenic micro-organisms. The selective participation of annexins in the endocytic pathways in DC suggests that these proteins could play distinct roles in the cellular events, leading to processing and presentation of different types of antigenic material.

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