Differential subcellular localization of CD86 in human PBMC-derived macrophages and DCs, and ultrastructural characterization by immuno-electron microscopy

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Keywords: cytoplasmic CD86

Abstract

We have previously reported the presence of a discrete reservoir of the costimulatory molecule CD86 in the cytoplasm of human monocytes freshly isolated from peripheral blood mononuclear cells (PBMC). In the current study, we have extended analysis of the subcellular localization of this molecule to in vitro PBMC-derived dendritic cells (DCs) and macrophages. In a sub-population of DCs, we observed by confocal microscopy an intracellular focal concentration of CD86 that bore striking similarities to that previously reported in monocytes. Further analyses revealed that this intracellular CD86 was not localized to the Golgi apparatus, MHC II compartments or endocytic structures, and required intact microtubules to retain structural integrity. A similar concentration of CD86 was not present in PBMC-derived macrophages. Electron microscopy revealed two distinct DC phenotypes containing either sparse or abundant cytoplasmic vesicles, and CD86 was found to be concentrated within the vesicular compartment of this latter phenotype. Collectively, these data not only identify and characterize a novel CD86-containing cytoplasmic compartment in human PBMC-derived DCs, but also define micro-structurally distinct DC subsets that differentially concentrate CD86 within cytoplasmic vesicles. Although the functional significance of these observations remains to be established, available evidence supports the conclusion that the focal concentration of CD86 is a storage reservoir that facilitates rapid deployment of this molecule to the DC surface when increased costimulatory capacity is required.

Introduction

The costimulatory molecules, CD80 and CD86, are type I integral membrane glycoproteins that are expressed on the surface of activated T lymphocytes, B lymphocytes and antigen-presenting cells (APC) (1,2). Dissection of the individual functions of these molecules in the initiation and regulation of immune responses is complicated by a shared capacity to function as low and high affinity ligands for the counter-receptors CD28 and CTLA-4, respectively (3,4). Although differences in amino acid sequence and domain structure provide a molecular basis for distinctly different actions, the spatial and temporal patterns of expression of these molecules are likely to be similarly important. CD86 exhibits a broader pattern of constitutive expression, being present at substantial levels on resting monocytes (2,5,6), macrophages (7) and dendritic cells (5,8), while CD80 expression is absent or low on the same cells in the absence of stimulation (5,7–9). Such patterns provide potentially important clues to function. For example, constitutive expression of CD86 on APCs is consistent with the established view that this molecule acts early in the initiation of an immune response.
response (1,10,11), but is also consistent with the more recent hypothesis that constitutive expression of costimulatory molecules on APCs may actually act to suppress T cell activation and maintain self-tolerance by sustaining a population of regulatory T cells (12). The current study further characterizes the spatial patterns of CD80 and CD86 expression in macrophages and DCs, thereby providing a basis for further insights into the function of these costimulatory molecules in APCs.

We have previously used retrovirus-mediated gene transfer to express CD86 in CEM cells, a human lymphoid cell line. In addition to cell-surface expression of CD86, we observed an unexpected intracellular focal concentration of this molecule (6). Extension of these studies to analysis of endogenous CD86 expression in normal human PBMC revealed a similar intracellular focal concentration in monocytes, but not in B or T lymphocytes, despite significant cell-surface expression following in vitro stimulation. The focal concentration of CD86 in monocytes was not due to storage within the Golgi apparatus, or with intact microtubules to retain structural integrity. Furthermore, when monocytes were stimulated by in vitro culture, cell-surface CD86 increased synchronously with depletion of cytoplasmic CD86. We therefore hypothesized that this previously undescribed focal concentration of CD86 in monocytes (blood APCs) might function as a storage reservoir of CD86, ready for rapid deployment to the cell surface.

In the current study, we set out to determine whether this discrete intracellular reservoir of CD86 is unique to monocytes or whether it is also present in APCs derived from this population. Established methods were used to differentiate adherent fraction human peripheral blood monocytes into DCs or macrophages by in vitro culture in media with or without interleukin 4 (IL-4) and granulocyte-macrophage colony-stimulating factor (GM-CSF), respectively (13–15). DCs derived in this manner have been shown to be potent stimulators of allogeneic T-lymphocyte proliferation (16,17). Here we present evidence for the existence of a novel CD86-containing storage compartment with distinct ultrastructure that may define a functionally distinct PBMC-derived DC subset. The biological implications of these findings are discussed.

Methods

In vitro differentiation of macrophages and DCs from peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) from consenting normal donors were isolated on Ficoll-Paque™ gradients according to the manufacturer's protocol (Pharmacia Biotech, Sweden). PBMCs were diluted in RPMI (Life Technologies, Grand Island, NY) containing penicillin (50 IU/ml, Life Technologies), streptomycin (50 μg/ml, Life Technologies), glutamine (2 mM, Life Technologies) and 10% (v/v) heat inactivated fetal bovine serum (Life Technologies), referred to hereafter as RPMI–FBS. Cells were dispensed in 3 ml volumes of DMEM–FBS into each well (1 × 10^7 cells/well) of a 6-well tissue culture plate (Falcon, BD Biosciences, Mountain View, CA) and incubated for 2–3 h at 37°C in a humidified atmosphere containing 5% CO₂. Non-adherent cells were removed by washing three times with warm PBS (without calcium and magnesium, Life Technologies). The adherent cell fractions were then incubated for 6 to 8 days in either RPMI–FBS or RPMI–FBS supplemented with 1000 U/ml GM-CSF (R&D, Bioscientific, Australia) and 500 U/ml IL-4 (R&D) to yield macrophages and DCs, respectively. Half volume media changes were performed twice daily. When required, DC maturation was induced during the last 24 to 48 h by supplementing DC media with TNF-α (100 U/ml, R&D). Cells were harvested and washed in PBS before examination by flow cytometry (FACS), immunofluorescent confocal microscopy or electron microscopy (EM).

Flow cytometry and immunocytochemistry

Flow cytometry was performed on a FACScan cytometer (Becton Dickinson) according to the manufacturer's protocols. Macrophages and DCs were gated using forward scatter (FSC) and side scatter (SSC) to exclude contaminating lymphocytes from the final analysis. Immunofluorescent confocal microscopy was performed using a Leica (Germany) CLSM confocal microscope equipped with an argon/krypton laser, as described (6). Cells were labeled for flow cytometry and immunofluorescent confocal microscopy with saturating quantities of the following murine antibodies: anti-CD1a–RD, anti-CD1a–PE, anti-CD14–FITC, anti-BB-1 (CD80), anti-CD83–RPE, anti-CD86, anti-CD86–FITC (BD Biosciences), anti-CD14–PE, anti-MHC II (HLA-DR, DQ, DR), Dako Australia Pty Ltd), anti-Golgi 58K protein (Sigma-Aldrich, St Louis, MO), anti-β-tubulin (SM162, Sternberger Monoclonals, Baltimore, MD) and cathepsin D (Chemicon, CA). Unconjugated murine antibodies were detected with fluorescein (FITC)-conjugated goat anti-mouse Ig (BD Biosciences). In immunofluorescent confocal microscopy studies, rhodamine red-X-conjugated goat anti-mouse Ig (Jackson ImmunoResearch Labs, PA) or FITC-conjugated donkey anti-mouse Ig (Jackson ImmunoResearch) were used to detect the relevant unconjugated antibodies. Goat polyclonal antibodies EEA-1 (Santa Cruz) and LAMP-2 (Santa Cruz) were detected using rhodamine red-X-conjugated donkey anti-goat Ig (Jackson Immuno Research). The patterns of anti-EEA-1 and anti-LAMP-2 antibodies in immunofluorescent confocal microscopy colocalization experiments were compared to the labeling pattern of cytoplasmic CD86 in a minimum of 40 consecutive DCs exhibiting the focal concentration of CD86 protein.

Disruption of the Golgi apparatus by Brefeldin A and microtubules by nocodazole treatment

Dendritic cells were treated with 0.7 mM Brefeldin A (BFA, Sigma-Aldrich) for 30 min at 37°C to disrupt the Golgi apparatus, or with 50 μM nocodazole (Sigma-Aldrich) for 60 min at 37°C to disassemble microtubules, as previously described (6). After washing, cell samples were attached to slides coated with Cell Tak® (Becton Dickinson), fixed in ice-cold 4% (v/v) paraformaldehyde (pH 7.4) and methanol prior to immunolabeling with anti-CD86, anti-Golgi 58K protein, anti-tubulin or isotype control, as appropriate, followed by secondary FITC-conjugated goat anti-mouse antibody. The immunofluorescent confocal microscopy localization patterns
were compared to untreated control DCs immunolabeled with the relevant antibodies.

**Inhibition of protein synthesis by treatment with cycloheximide**

PBMC-derived DCs were treated with 20 μg/ml cycloheximide (ICN Biomedicals, Ohio) for 3 h at 37°C to inhibit protein synthesis. Cell samples were washed in PBS, cyto spun and fixed prior to immunolabeling with FITC-conjugated anti-CD86 and anti-MHC II (visualized using rhodamine red-X-conjugated goat anti-mouse antibody). Parallel cell samples were immunolabelled for FACS analysis with anti-CD1a, anti-CD83, anti-CD86 and anti-MHC II. The immunofluorescent confocal microscopy localization patterns and FACS profiles were compared to control untreated DCs immunolabeled with the same antibodies. Antibody specificity was confirmed using appropriate isotype controls.

**Immunoelectron microscopy on cryopreserved sections of CEM cells**

CEM cells genetically modified to express CD86 (6) were lightly fixed in PBS containing 4% (v/v) formaldehyde and 0.1% (v/v) glutaraldehyde and prepared for immunoelectron microscopy. Briefly, cells were dispersed into agarose, sectioned and cryoprotected in dimethyl formamide for 30 min at −70°C. Frozen samples were freeze-substituted in methanol containing 0.5% (w/v) uranyl acetate at −80°C for 48 h. The solvent was replaced with Lowcryl resin (ProSciTech) overnight and the resin polymerized with UV light for 48 h at −45°C. Ultra-thin sections (60 nm) were placed in grids for immunolabeling with anti-CD86 followed by anti-mouse gold conjugate (5 nm) (Sigma) and examined using a Philips CM 10 electron microscope.

**Preparation of resin-embedded ultra-thin sections for transmission electron microscopy**

Dendritic cells were fixed in a modified Karnovsky fixative [2.5% (v/v) glutaraldehyde, 2.5% (v/v) formaldehyde (freshly prepared from paraformaldehyde) in 0.1 M 3-[N-morpholino]-propane-sulfonic acid (MOPS) buffer (Sigma)] for 1 h. After washing in 0.1 M MOPS buffer, cells were post-fixed in 2% (w/v) osmium tetroxide, dehydrated in an ethanol series and embedded in Spurr epoxy resin. The resin was polymerized at 70°C for 10 h and ultra-thin (60 nm) sections prepared.

**Preparation of cryopreserved sections for light microscopy and immuno-electron microscopy**

Dendritic cells were lightly fixed in PBS (without calcium and magnesium) containing 2% (v/v) formaldehyde (freshly prepared from paraformaldehyde) and 0.05% (v/v) glutaraldehyde for 1 h at 4°C. After washing in PBS, cells were pelleted in gelatin and cryoprotected in 1.7 M sucrose with 15% (w/v) polyvinyl pyrrolidone (MW 10 000) (18) overnight at 4°C. Cells were then frozen in liquid nitrogen and subsequently sectioned using a Leica Ultracut S/FCS cryo-ultramicrotome.

Cryopreserved DCs were sectioned progressively either as semi-thin (0.5 μm) or ultra-thin (60 nm) sections and the sequential sections examined. Semi-thin sections were cut in order to assess the cell sampling as well as the efficiency and specificity of anti-CD86 gold immunostaining. Thawed semi-thin sections were immunolabeled with anti-CD86 antibody and visualized with gold (10 nm) conjugated secondary antibody (British BioCell) and silver enhanced (British BioCell) (brown colour), followed by counter-staining to show the blue cytoplasm and nucleus. Sections were examined using a Leica DML microscope (Leica Microsystems, Gladesville, NSW, Australia) and images were collected by a cooled CCD camera (SPOT 2 camera, SciTech Pty Ltd, Victoria, Australia) and digitized. Cells were examined for the presence or absence of a focal concentration of CD86.

Ultra-thin sections of DCs cut directly after the semi-thin samples described above were collected onto gilded nickel grids (coated with a Formvar/Pioloform film) and blocked overnight in 20% (v/v) FBS in PBS. Sections were then labeled with anti-CD86 antibody followed by a secondary anti-mouse antibody conjugated to 10 nm gold particles (British BioCell). Sections were embedded in methyl cellulose/uranyl acetate solution prior to examination using a Philips CM 120 BioTwin or CM10 transmission electron microscope at 80 kV. Ultra-thin sections of cells with sparse and vesicular phenotypes were scored for the presence of CD86–gold in cytoplasmic vesicles. DCs with fewer than two particles of CD86–gold in any vesicle were defined as moderately stained. Those with vesicles containing more than two particles were defined as intensely stained.

**Results**

**Derivation and immunophenotypic characterization of PBMC-derived macrophages and DCs**

Macrophages or DCs were derived from the adherent fraction of healthy donor PBMC by 7 days culture in medium (RPMI-FBS) alone, or medium supplemented with GM-CSF and IL-4, respectively. The differentiation of macrophages from monocytes was associated with a significant increase in cell size as judged by forward and side scatter FACS plots, but there were no qualitative changes in the pattern of immunophenotypic markers examined (Fig. 1A and Table 1). The differentiation of DCs from monocytes was associated with an increase in cell size, loss of the monocyte-macrophage marker CD14, and appearance of CD1a and CD80 expression. All cell types constitutively expressed CD86 and MHC II. Treatment of DC with TNF-α resulted in the appearance of CD83 on the cell surface, indicative of maturation (Fig. 1B and Table 1).

A prominent focal concentration of CD86 is observed in DCs, but is absent in macrophages.

Intracellular expression of CD86 in monocytes, macrophages and DCs was analyzed by immunofluorescent confocal microscopy after immunolabeling with anti-CD86 antibody. As previously reported (6), CD86 was expressed on the cell surface of freshly isolated peripheral blood monocytes, with a proportion also containing a focal concentration of CD86 in the cytoplasm (Fig. 2A) (6–31% range, 19.8 ± 8%; mean ± SD, in 10 samples). Following differentiation of monocytes into macrophages, cell-surface CD86 expression persisted but the focal concentration of cytoplasmic CD86 was no longer evident (Fig. 2B). In distinct contrast, differentiation of
Intracellular localization of CD86 in human DCs

Monocytes into DCs was associated with both increased cell-surface CD86 expression, and the presence of a large (up to 10 μm in diameter) prominent focal concentration of cytoplasmic CD86. This focal concentration of CD86 was observed in a proportion of DCs (Fig. 2C) ranging from 5 to 33% (14.7 ± 8%: mean ± SD) in 15 independent preparations from five different PBMC donors. Variability within and between donors was similar (not shown). At high magnification, the focal concentration of CD86 exhibited a densely packed, punctate, possibly vesicular pattern (Fig. 2D). While intracellular CD80 was detected in PBMC-derived macrophages and DCs, neither cell type exhibited a distinctive focal concentration of this molecule (data not shown).

The focal concentration of CD86 in DCs is not associated with the Golgi apparatus and relies on intact microtubules for structural integrity.

The previously reported intracellular reservoir of CD86 in peripheral blood monocytes was shown not to be associated with the Golgi apparatus and required intact microtubules to retain structural integrity (6). To establish whether the focal concentration of CD86 observed in DCs had similar properties, immunofluorescent confocal microscopy analysis of DCs labeled simultaneously with anti-CD86 and anti-Golgi 58K protein antibodies was performed. This analysis revealed that the focal concentration of CD86 observed in a proportion of DCs (Fig. 3A) did not co-localize with the Golgi apparatus (Fig. 3B). This observation was confirmed by failure of BFA, an agent that disrupts the Golgi apparatus (Fig. 3D), to exert an effect on the focal concentration of CD86 (Fig. 3C). Nocodazole treatment, in concert with anti-β-tubulin and anti-CD86 antibody labeling, was used to examine the dependence of the focal concentration of CD86 on the microtubular network for retention of structural integrity. The normal microtubule pattern observed in untreated DCs immunolabeled with anti-β-tubulin antibody (Fig. 3E) was no longer evident after nocodazole treatment (Fig. 3F), and the intracellular focal concentration of CD86 in DCs (Fig. 3G) was similarly disrupted after nocodazole treatment (Fig. 3H).

The focal concentration of CD86 in DCs is not associated with MHC class II proteins and persists in DCs after maturation with TNF-α.

Because MHC II-peptide complexes, newly formed in late endosomes or lysosomes, have been reported to selectively

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**Table 1. Immunophenotype of human PBMC-derived monocytes, macrophages and dendritic cells**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Monocytes</th>
<th>Macrophages</th>
<th>Dendritic cells</th>
<th>Mature dendritic cells</th>
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<tr>
<td>CD1a</td>
<td>0 (3)</td>
<td>0 (6)</td>
<td>76 ± 29 (8)</td>
<td>17 (1)</td>
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<tr>
<td>CD14</td>
<td>91 ± 5 (15)</td>
<td>94 ± 5 (6)</td>
<td>0 (7)</td>
<td>0 (1)</td>
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<tr>
<td>CD80</td>
<td>0 (4)</td>
<td>4 ± 1 (4)</td>
<td>88 ± 20 (11)</td>
<td>88 ± 22 (4)</td>
</tr>
<tr>
<td>CD83</td>
<td>0 (3)</td>
<td>2 ± 4 (4)</td>
<td>12.5 ± 6 (10)</td>
<td>66 ± 16 (5)</td>
</tr>
<tr>
<td>CD86</td>
<td>91.9 ± 8 (15)</td>
<td>86 ± 20 (6)</td>
<td>72.5 ± 21 (12)</td>
<td>98 ± 1.7 (5)</td>
</tr>
<tr>
<td>MHCII</td>
<td>89.1 ± 7 (6)</td>
<td>98 ± 1 (5)</td>
<td>97 ± 1.8 (12)</td>
<td>94 ± 2.9 (4)</td>
</tr>
</tbody>
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Percentage of cells positive for indicated antibody, mean ± SD (n)

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**Fig. 1. Morphology and immunophenotype of PBMC-derived monocytes, macrophages and DCs by FACScan analysis.** Scatter plots, indicative of cell morphology, and histogram profiles (unshaded) of cell-surface molecule expression are shown. Labeling with relevant isotype control antibodies is shaded. Adherent fraction PBMC-derived monocytes (left column), macrophages (middle column) and DC (right column) (A). PBMC-derived DC before (left column) and after TNF-α treatment (right column) (B).
accumulate with intracellular CD86 in murine DCs (19), we examined whether the intracellular focal concentration of CD86 was co-localized with MHC II molecules. Monocyte-derived DCs were immunolabeled simultaneously with anti-CD86 and anti-HLA-DP,DQ,DR antibodies and visualized by immunofluorescent confocal microscopy. MHC II molecules were universally observed on the cell-surface of DC; however, the pattern of MHC II expression in the cytoplasm (Fig. 3J) was distinctly different to that of CD86 (Fig. 3I). Diffuse aggregates of MHC class II molecules were faintly discernible in the cytoplasm (Fig. 3K) but were not observed at the same focal plane as the cytoplasmic concentration of CD86 (Fig. 3J). Furthermore, following maturation of DCs by treatment with TNF-α, cytoplasmic MHC II molecules were no longer evident and MHC II cell-surface expression was more intense (Figs 3L and 1B). In contrast, the intracellular focal concentration of CD86 persisted in a subpopulation of TNF-α matured DCs (5.2 ± 2.8, n = 6) despite the marked increase in cell-surface CD86 expression (Fig. 1B). In short, while CD86 and MHC II molecules were observed to co-localize at the cell surface, the intracellular compartmentalization and trafficking of these molecules in PBMC-derived DC appears to be distinctly different.

The focal concentration of CD86 in DCs is not associated with endocytic structures

Although there was no evidence of CD86 colocalization with cytoplasmic MHC II, we investigated whether the focal concentration of CD86 was associated with specific endocytic structures. As an initial step, DCs were immunolabeled simultaneously with anti-CD86 and anti-HLA-DP,DQ,DR antibodies and visualized by immunofluorescent confocal microscopy. MHC II molecules were universally observed on the cell-surface of DC; however, the pattern of MHC II expression in the cytoplasm (Fig. 3J) was distinctly different to that of CD86 (Fig. 3I). Diffuse aggregates of MHC class II molecules were faintly discernible in the cytoplasm (Fig. 3K) but were not observed at the same focal plane as the cytoplasmic concentration of CD86 (Fig. 3J). Furthermore, following maturation of DCs by treatment with TNF-α, cytoplasmic MHC II molecules were no longer evident and MHC II cell-surface expression was more intense (Figs 3L and 1B). In contrast, the intracellular focal concentration of CD86 persisted in a subpopulation of TNF-α matured DCs (5.2 ± 2.8, n = 6) despite the marked increase in cell-surface CD86 expression (Fig. 1B). In short, while CD86 and MHC II molecules were observed to co-localize at the cell surface, the intracellular compartmentalization and trafficking of these molecules in PBMC-derived DC appears to be distinctly different.

Two distinct patterns of ultrastructure were observed in the cytoplasm of DCs examined using transmission electron microscopy (TEM)

To further characterize the focal concentration of CD86 in DCs identified by immunofluorescent confocal microscopy, conventional TEM was undertaken on resin embedded sections.
Cells in which the plane of the EM section passed through the axis of the cell, including the nucleus, were evaluated (n > 100). In a minority of DCs, ~10%, there was a prominent aggregation, up to 10 μm across, of densely packed vesicles (>30 in the plane of the EM section) in the cytoplasm adjacent to the nucleus (Fig. 6A). The remaining DCs contained fewer vesicles distributed more sparsely throughout the cytoplasm (Fig. 6B). The vesicles were membrane bound and round to ovoid in shape. A proportion also exhibited double membranes (Fig. 6C, inset) and/or contained one or more smaller vesicular structures of variable electron density (Fig. 6C).

Immunoelectron microscopy evaluation of CD86 subcellular localization in DCs

In the process of developing a suitable immunoelectron microscopy protocol for detecting CD86, we used genetically modified CEM cells (L86SN-CEM) expressing CD86 with the view of performing similar immunoelectron microscopy studies in DCs. The genetically modified CEM cells were selected for high expression of surface CD86 with the important additional advantage that every L86SN-CEM cell constitutively expressed the focal concentration of CD86 thereby increasing the probability of observing cytoplasmic CD86–immunogold labeling. Intriguingly, in one section, we detected evidence of a CD86–immunogold tracking extending from near the nuclear membrane (Fig. 7A and C) out to the plasma membrane (Fig. 7A and B). The dense gold labeling appearing to lie beyond the plasma membrane (Fig. 7B) may have been attached to a cellular projection lying outside the plane of section. No immunogold was evident in control LXSN-CEM cells immunostained in the identical manner (not shown). Although CD86–immunogold was not associated with cytoplasmic organelles in L86SN-CEMs, the tracking of the substantial CD86–immunogold suggests trafficking of CD86 along a cytoplasmic pathway.

Having developed a suitable protocol for the detection of CD86 by immunoelectron microscopy, we applied this technique to the detection of this molecule in DCs. The DCs to be examined were first divided into equivalent aliquots. Analysis of one aliquot by immunofluorescent confocal microscopy revealed that 9.2% of DCs (73 of 793 cells examined) contained a focal concentration of CD86. The other aliquot was prepared for immunoelectron microscopy studies and preliminary CD86 antibody characterization was performed using semi-thin, thawed cryosections and silver enhanced immunogold staining. On light microscopy examination, the silver enhanced immunogold CD86 was observed as a focal concentration in the cytoplasm of 8.6% of cells (44 of 513), a result remarkably consistent with that obtained by immunofluorescent confocal microscopy analysis.

This second aliquot was processed further to examine whether the accumulation of vesicles observed using conventional TEM, which appeared very similar in size and location to the focal concentration of CD86, did indeed label with CD86–gold. Ultra-thin cryosections, taken adjacent to the semi-thin sections described above, were immunogold labeled for CD86 and analyzed by TEM. Strikingly, immunogold particles were detected in the small subset of DCs with the dense vesicular phenotype (Fig. 8A). In 21 consecutive DCs of this
phenotype examined, moderate ($n = 9$) to intense ($n = 12$) CD86 labeling was observed in the vesicular compartment (Fig. 8B). Background staining was minimal. In contrast, in nine consecutive DCs with the predominant sparse vesicular phenotype examined, cytoplasmic gold particles were rarely observed (Fig. 8B).

**Discussion**

In a previous study, we reported the presence of a focal concentration of CD86 protein in the cytoplasm of a proportion of human peripheral blood monocytes, and provided evidence that this protein reservoir serves to allow rapid deployment of CD86 to the cell surface (6). In the current study, we set out to further characterize this cytoplasmic reservoir of CD86, and to establish whether it is also present in monocyte-derived macrophages and DCs, which are known to constitutively express CD86 (1,9,22). Using established methods, human peripheral blood monocytes were cultured and differentiated into macrophages and DCs, respectively (5,14,23). Consistent with well established patterns of cell-surface CD80 and CD86 expression on APCs, monocytes and macrophages were found to constitutively express low levels of CD80 and moderate levels of CD86, while both costimulatory molecules were expressed at high levels on DCs. Investigation of intracellular CD86 expression revealed that a proportion of DCs, but not macrophages, contain a substantial intracellular focal concentration of CD86 (up to 10 $\mu$m in diameter), similar to that previously reported in monocytes (6). While cytoplasmic CD80 could be detected in both cell types, the distribution was more punctate and diffuse.

Characterization of the focal concentration of CD86 in DCs revealed features previously observed in monocytes and CEM cells genetically modified to express this molecule i.e., the focal concentration of CD86 was not localized to the Golgi complex and required intact microtubules for maintenance of structural integrity. Treatment of DCs with the protein synthesis inhibitor, cycloheximide, resulted in rapid disappearance of the abundant cytoplasmic CD86 and a concurrent increase in cell-surface CD86, providing further evidence for antegrade transport of CD86 from an intracellular compartment to the cell-surface membrane.

In murine DCs, CD86 has been shown to selectively accumulate with MHC II in lysosome marker-negative vesicles termed CIIVs (19). MHC II molecules in DCs have also been shown to target endocytic compartments (MIICs) (24–26) and recent reports have demonstrated that newly formed immunogenic MHC II–peptide complexes are transported from late endosomal (27) and lysosomal compartments (28) to the cell surface of DCs via tubular endosomes that fuse directly with the plasma membrane. Our data indicate that cytoplasmic CD86 in dendritic cells is not associated with MHC II molecules or with endocytic structures. Additionally, the diffuse cytoplasmic distribution of MHC II disappeared in DCs after maturation with TNF-α, as previously reported (15), while the focal concentration of CD86 remained unaffected. These data do not preclude association between CD86 and endocytic pathways in human PBMC-derived DCs, but collectively provide strong evidence that the focal concentration of CD86 is not contained within an endosomal compartment.
Collectively, our data are consistent with the possibility that cytoplasmic CD86 is associated with distinct ultrastructure in DCS. Conventional TEM analysis of DC sections revealed two distinctly different cellular cross-sections, with the majority containing a scattering of mitochondria, Golgi, smooth and rough endoplasmic reticulum and very few cytoplasmic vesicles. In marked contrast, the remaining DCs contained a large cluster of densely packed vesicles which occupied a substantial area of the cytoplasm. Moreover, the juxta-nuclear location and the cross-sectional area occupied by these vesicular clusters correlated with that of the focal concentration of CD86 in the immunofluorescent confocal microscopy images, indicating that these could be the same structure. This conclusion was supported by immuno-electron microscopy studies which revealed that cytoplasmic CD86 expression was confined, almost exclusively, to the vesicles of DCs exhibiting the dense vesicular phenotype.

While the morphological features of the vesicles observed in the TEM and immunogold studies did not provide direct clues to the identity of the CD86-containing compartment, it was possible to preclude several possibilities. The morphology was inconsistent with a tubo-vesiculur structure, was not multilaminar as observed in MIICs (26) and was distinctly different to that of Birbeck-like granules that have previously been reported in monocyte-derived DCs (29). Birbeck-like granules have a characteristic ultra-structure consisting of two concentric crescent-shaped membranes separated by dense matrix (29,30). It is therefore reasonable to hypothesize that the densely clustered CD86-containing vesicles observed in a subset of monocyte-derived DCs represent a novel compartment that has evolved as an APC-specific adaptation to the demands of CD86-mediated costimulation (31).

No evidence of CD86 tracking was detected in the cytoplasm of DCs as was observed in the genetically modified CEM cells (L86SN-CEM) used to work-up the immuno-electron microscopy protocol. Failure to detect CD86 tracking, however, does not preclude the existence of this phenomenon in DCs as there is a low probability of capturing such a track in the plane of an ultra-thin cryosection where only a small percentage of cells contain the intracellular reservoir of CD86. Further analyses will be required to explore this possibility.

Monocytes, members of the mononuclear phagocyte system, exhibit weak immunostimulatory capacity compared to DCs (32) and have been primarily regarded as a source of peripheral macrophages and DCs. Recent characterization of human peripheral blood monocytes has revealed the existence of unique sub-populations with specific immunoregulatory properties and DC-like characteristics (33). There has also been growing interest in identifying DC subsets with potential for therapeutic exploitation (34,35). Correlation of microstructural and functional properties will facilitate increasingly precise characterization of DC subsets and their biological significance (34,36). It is possible that the presence of a focal concentration of CD86 in monocytes and monocyte-derived DCs simply reflects a cellular maturation/activation state or, more significantly, defines a functionally distinct subset within the DC population. It also remains to be established whether the monocyte-derived DCs containing the focal concentration of CD86 arise from the subset of monocytes observed with intracellular CD86.

Fig. 6. Ultrastructural examination of resin embedded DC. Two distinctly different phenotypes were observed; DCs with a dense juxta-nuclear aggregation of cytoplasmic vesicles (A) and DCs with a paucity of cytoplasmic vesicles (B). The area marked with an asterisk in (A) has been further magnified (C). The inset in (C) shows single membrane and double membrane (v) vesicles. N: nucleus; PM: plasma membrane. In (A and B) and (C), the scale bars represent 2 μm and 1 μm, respectively.
Collectively, our studies demonstrate that CD86 is not only differentially expressed but is also differentially localized in human hematopoietic cells. The biological significance of the observations linking the dense cytoplasmic vesicles with the cytoplasmic store of CD86 protein in DCs is consistent with the hypothesis that this compartment acts as a reservoir of pre-formed CD86 available for rapid deployment to the cell surface. The presence of this reservoir in DCs, but not in macrophages, probably reflects the biological function of these cells and provides further insight into the temporal regulation of APC function in the cell-mediated immune response.

Acknowledgements
We thank Debrah Birch (EM Unit, Macquarie University, NSW 2091) for her expert electron microscopy.

Abbreviations

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<th>Abbreviation</th>
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<tr>
<td>APC</td>
<td>antigen-presenting cells</td>
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<td>DCs</td>
<td>dendritic cells</td>
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<td>FSC</td>
<td>forward scatter</td>
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<td>GM-CSF</td>
<td>granulocyte-macrophage colony-stimulating factor</td>
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<td>interleukin 4</td>
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<td>MOPS</td>
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References


Fig. 7. Ultra-thin cryosection of a L86SN-CEM cell immunolabeled with anti-CD86 antibody gold (5 nm). An almost continuous track of CD86–gold can be seen extending through the cytoplasm from close to the nuclear membrane out to the cell-surface membrane (A open arrow). Examination, at higher magnification of the areas indicated by the solid (C) and open (B) arrows in (A) reveal the substantial level of CD86 gold labeling. N: nucleus; PM: plasma membrane; CP: cellular projection. No immunogold was detected in control LXSN-CEM cells immunostained in an identical manner (not shown). Scale bars in (A) and (B and C) represent 364 nm and 95 nm, respectively.

Fig. 8. Appearance by TEM and quantitative analysis of CD86–gold in cytoplasmic vesicles of DCs. An ultra-thin cryosection of a DC, of the dense vesicular phenotype, immunolabeled with anti-CD86 antibody gold (10 nm) reveals a concentration of CD86–gold within a vesicle and lower levels of labeling immediately adjacent to the vesicle (A). Scale bar represents 200 nm. The number of cytoplasmic vesicles containing CD86–gold was examined in a total of 30 DCs; nine of the sparse vesicular phenotype (open bars) and 21 of the dense vesicular phenotype (hatched bars) (B).
Intracellular localization of CD86 in human DCs


