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Identification of a Dynamic Intracellular Reservoir of CD86 Protein in Peripheral Blood Monocytes That Is Not Associated with the Golgi Complex

Christine Smyth,* Grant Logan,* Ron P. Weinberger,‡ Peter B. Rowe,* Ian E. Alexander,† and Jason A. Smythe1*†

In the process of developing a cancer immunotherapy strategy, we have identified and characterized a novel intracellular reservoir of CD86 protein in peripheral blood monocytes. This observation emerged from studies aimed at using retrovirus vectors to genetically modify tumor cells to express the costimulatory proteins CD80 and CD86. Retrovirus-mediated expression of CD80 and CD86 in T lymphoblastoid CEM cells resulted in an unexpected intracellular focal concentration of both proteins in the genetically modified cells. By extending these studies to an analysis of CD80 and CD86 expression in PBMC, we observed that endogenous CD86 expression in peripheral blood monocytes also involved a similar intracellular focal concentration of the protein. The intracellular concentration of CD86 in monocytes was not due to storage within the Golgi apparatus, and required intact microtubules to retain structural integrity. Furthermore, as the intensity of CD86 fluorescence increased on monocytes as a function of time in vitro, the intracellular focal concentration correspondingly decreased. These results are consistent with antegrade CD86 transport from an intracellular reservoir to the cell surface membrane. In this report, we detail the intracellular and membrane localization studies with tumor cell lines and PBMC, and describe the temporal relationship between intracellular storage and trafficking of CD86 to the cell surface membrane in peripheral blood monocytes. We hypothesize that this intracellular reservoir allows rapid and sustained deployment of an important costimulatory molecule to the monocyte surface membrane during initiation and maturation of the cell-mediated immune response. The Journal of Immunology, 1998, 160: 5390–5396.

One of the primary objectives in cancer research is to thoroughly understand the mechanisms by which tumors evade recognition and rejection by cells of the human immune system. By understanding these processes it may be possible to develop therapeutic strategies for manipulating the immune system to enhance or augment antitumor effector functions. Although the idea of immune augmentation is not new (reviewed in Ref. 1), recent advances in our understanding of T lymphocyte activation and costimulation have provided a sound basis for developing this type of tumor-specific immunotherapy strategy (2–10). We are focussing on developing an immunotherapy for the treatment of pediatric cancer based on using retroviral vectors to genetically modify tumor cells to express CD80 and CD86.

CD80 and CD86 are type I integral membrane glycoproteins and members of the Ig supergene family found on the surface of activated T lymphocytes, B lymphocytes, and APC. During the process of lymphocyte activation by costimulation, CD80 and CD86 function as low affinity ligands, or counterreceptors for CD28 and high affinity counterreceptors for CTLA-4. Functionally, CD86 appears to be important in the initiation of a cell-mediated immune response, whereas CD80 is believed to be involved in the maintenance and/or moderation of the immune response (2, 3, 6–12).

The strategy we are pursuing is aimed at rendering the tumor cells capable of simultaneously providing an Ag-specific signal to an appropriate T lymphocyte, and a second costimulatory signal through the CD28 receptor molecule. The ultimate aim is to activate tumor-specific T lymphocytes without the need for APC, and the results of several in vivo studies with murine tumor models have already validated this hypothesis under certain conditions (5, 13–15). In developing this strategy, we initially focussed on evaluating retrovirus-mediated gene transfer (transduction) of CD80 and CD86 to human lymphoid (CEM), and nonlymphoid (HeLa) tumor cell lines. After genetically modifying the tumor cells, we observed an unexpected intracellular focal concentration of CD80 and CD86 in transduced CEM cells. Subsequent studies of endogenous CD80 and CD86 gene expression in PBMC revealed a strikingly similar intracellular focal concentration of CD86 protein in monocytes. This intracellular focus was not due to concentration of CD86 in the Golgi, and required intact microtubules to retain structural integrity. This report details the intracellular and membrane localization studies, and describes the temporal regulation of intracellular storage and trafficking of CD86 to the cell surface membrane of monocytes. We discuss the biologic implications of these findings, and hypothesize that this intracellular concentration of CD86 in monocytes represents a functional reservoir of protein. This reservoir may be important in enabling the monocyte to respond to stimulation by rapid and sustained deployment of CD86 to the cell surface membrane.

Materials and Methods

Production of ecotropic and amphotropic retroviruses and transduction of tumor cell lines

Psi-2(16), PA317 (American Type Culture Collection (ATCC), Rockville, MD; CRL 9078), and HeLa (ATCC CCL-2) cells were maintained in DMEM, and CEM (ATCC CCL 119 CCRF-CEM) cells were maintained...
in RPMI supplemented with 10% heat-inactivated bovine calf serum (Star-
rate, Bethunra, Australia), penicillin (50 IU/ml), streptomycin (50 µg/ml),
and 2 mM glutamine, and cultured at 37°C in a 5% CO₂ humidified
incubator. Human CD80 (B7-1) cDNA (GenBank accession no. M27533) and
CD86 (B7-2) cDNA (GenBank accession no. L25259) were kindly pro-
vided by Dr. Gordon Freeman (Dana-Farber Cancer Institute, Harvard
Medical School, Boston, MA) in pCDM8 (2, 8). The CD80 and CD86
cDNA inserts were isolated after XhoI digestion and subcloned into the
XhoI site of the retrovirus vector plasmid pLXSN to generate plasmids
pL80SN and pL86SN, respectively. The integrity and orientation of the
cDNA inserts was confirmed by dyeodeoxy nucleotide sequencing.

Psi-2 and PA317 cells were selected in HAT (0.1 mM hypoxanthine,
0.05 mM aminopterin, 0.016 mM thymidine) and HT-medium (0.01 mM
hypoxanthine, 0.016 mM thymidine) before use for retrovirus packaging.
Psi-2 cells were transfected with 3 µg of pLXSN, pL80SN, or pL86SN
dNA using 10 µl of LipofectAMINE (Life Technologies, Grand Island,
NY) according to the manufacturer’s instructions. After selection in
DMEM supplemented with G418 (600 µg/ml active compound; Life Tech-
nologies) ecotropic retrovirus corresponding to LXSN, L80SN, or L86SN
was harvested, filtered through a 0.45-µm pore size filter, and used to
transduce PA317 cells. LXSN-, L80SN-, and L86SN-transduced PA317
cells were then G418 selected (600 µg/ml active compound), and amphi-
 tropic retrovirus producer clones obtained by limiting dilution. Amphi-
 tropec secretantants were assayed for viral titer and replication-competent
recombinant retrovirus as described (17, 18) and used to transduce CEM or
HeLa cells, generating L80SN-CEM, L86SN-CEM, L80SN-HeLa, and
L86SN-HeLa cells. Each transduced tumor cell population was then G418
selected, and clones derived by limiting dilution. Flow cytometry by FACS
and immunofluorescence confocal microscopy (ICM)2 were then used to
evaluate expression and cellular localization of CD80 and CD86.

Flow cytometry (FACS) and ICM

Flow cytometry was performed on a FACSscan cyrometer (Becton Dick-
son, Mountain View, CA) according to the manufacturer’s protocols.
ICM was performed using a Leica (Deerfield, IL) CLSM confocal micro-
scope as described (19). Murine Abs used were anti-BB-1 (CD80) (CAM-
Folio; Becton Dickenson), CD86 (PharMingen, San Diego, CA), CD4-
FITC, CD8-PE, CD3-FITC, CD19-PE, CD28 (Becton Dickenson), HLA-
ABC (Silenus, Paris, France), CD14-PE (Dako, Carpinteria, CA),
Nucleolus AE-3 (Biosdesign, New York, NY), Golgi 58K protein (Sigma,
St. Louis, MO), β-tubulin (SM162; Steremberger Monoclonals, Baltimore,
MD), CD4, and CD11b (Dako). Unconjugated Abs were visualized by
incubation with FITC-conjugated goat anti-mouse Ig (Becton Dickenson).
The mean fluorescence intensity (MFI) was calculated for each FACS pro-
file. All samples examined by ICM were coded until completion of each
experiment.

Disruption of the Golgi apparatus and microtubules by brefeldin
A (BFA) and nocodazole treatment

As appropriate, L86SN-CEM cells or peripheral blood monocytes were
treated with 0.04 µM BFA for 20 min at 37°C to disrupt the Golgi appa-
ratus, or with 50 µM nocodazole (Sigma) for 60 min at 37°C to disrupt
microtubules. The cells were then washed with PBS and samples settled
onto glass slides; fixed with paraformaldehyde and methanol; and stained
with Ab to CD86, anti-Golgi 58K protein, anti-β-tubulin, or an isotype
control, as appropriate, and the secondary FITC-conjugated goat anti-
mouse Ab. To reassemble the Golgi apparatus and microtubules, BFA-
or nocodazole-treated cells were washed in PBS and incubated for 30 or 60
min, respectively, at 37°C in PBS containing 10% FCS (Life Technologies)
to remove the compound, and samples then fixed and stained as described
above. All ICM localization patterns were compared with slides of un-
treated control cells.

PBMC isolation, fractionation, and stimulation in vitro

Heparin-treated blood from normal donors was diluted 1:1 with PBS, and
PBMC isolated by adherence to plastic, and T lymphocytes
were isolated by SRC rosetting as described (20). The monocyte-depleted,
nonrosetting population (containing predominantly B lymphocytes) was
also collected. An analysis of purity for each cell fraction was determined
by FACS. Purified monocytes were stimulated by in vitro culture on plastic
in DMEM (not a bone containing bovine calf serum). Cell surface local-
ization of CD4, CD14, HLA-ABC, CD80, CD11b, and CD86 was moni-
tored by FACS and intracellular expression of CD4, CD80, CD86, and
CD11b by ICM. Purified T lymphocytes were stimulated by preincubation
for 1 h with 5 ng/ml of OKT3 Ab (Janssen-Cilag, Sydney, Australia) and
then extensively washed and maintained in rIL-2-supplemented DMEM
medium. Cell surface localization of CD4, CD8, CD14, CD19, HLA-ABC,
CD80, and CD86 was analyzed on T and B lymphocytes by FACS before
and after stimulation, and intracellular expressions of CD80 and CD86
by ICM.

Results

ICM detection of an intracellular focal concentration of CD80
and CD86 in retrovirus-transduced CEM cells. ICM analysis of CD80 and
CD86 protein localization within L80SN-CEM cells (A), L86SN-CEM
cells (B), L80SN-HeLa cells (C), and L86SN-HeLa cells (D) (single
images in the x-y plane). Consecutive ICM images (z-axis) of an L86SN-
CEM cell (E), and an L86SN-HeLa cell (F). Isotype-control Ab staining
was negative (not shown). Scale bar is 10 µm.

Before retrovirus transduction, CEM and HeLa cells were tested
by FACS analysis and ICM and shown to be negative for endog-
ous CD80 or CD86 expression (not shown). FACS analysis of
stable L80SN-CEM, L86SN-CEM, L80SN-HeLa, and L86SN-
HeLa clones revealed high levels of CD80 or CD86 cell surface
expression, as expected. ICM analysis of L80SN-CEM
and L86SN-CEM cells also revealed a prominent intracellular foc-
hal con UC concentration of CD80 (Fig. 1A) and CD86 (Fig. 1B),
respectively. This intracellular focal concentration of CD80 or CD86
protein was present in all L80SN-CEM and L86SN-CEM clones
examined. Fluorescence staining was not observed in the LXSN
vector-transduced control CEM cells, or after isotype-control Ab
staining (not shown).

A panel of L80SN-HeLa and L86SN-HeLa cell clones was also
examined for surface and intracellular localization of CD80 and
CD86 protein. In contrast to the results obtained with transduced
CEM cells, the transduced HeLa cell clones demonstrated strong
cell surface membrane localization of CD80 or CD86, but no in-
tracellular focal concentration of either protein (Fig. 1, C and D,
respectively). A series of ICM images of immunolabeled cells
were acquired in the plane (x-y-axis) of the slide throughout the

2 Abbreviations used in this paper: ICM, immunofluorescence confocal microscopy;
MFI, mean fluorescence intensity; BFA, brefeldin A.

FIGURE 1. ICM detection of an intracellular concentration of CD80
and CD86 in retrovirus-transduced CEM cells. ICM analysis of CD80 and
CD86 protein localization within L80SN-CEM cells (A), L86SN-CEM
cells (B), L80SN-HeLa cells (C), and L86SN-HeLa cells (D) (single
images in the x-y plane). Consecutive ICM images (z-axis) of an L86SN-
CEM cell (E), and an L86SN-HeLa cell (F). Isotype-control Ab staining
was negative (not shown). Scale bar is 10 µm.
Characterization of the intracellular focus of CD86 protein in L86SN-transduced CEM cells. Shown are ICM analyses of (A) L86SN-CEM cells immunostained with nucleolus-specific Ab AE-3 (left), anti-CD86 Ab (center), or both Abs simultaneously (right); (B) L86SN-CEM cells immunostained with Golgi-specific Ab (left), anti-CD86 Ab (center), or both simultaneously (right); (C) L86SN-CEM cells immunostained with Golgi-specific Ab before treatment with BFA (left), after treatment with BFA (center), and after BFA treatment and washing (right); (D) L86SN-CEM cells stained with anti-CD86 Ab before treatment with BFA (left), after treatment with BFA (center), and after BFA treatment and washing (right); (E) L86SN-CEM cells stained with anti-β-tubulin Ab before treatment with nocodazole (left), after treatment with nocodazole (center), and after nocodazole treatment and washing (right); and (F) L86SN-CEM cells stained with anti-CD86 Ab before treatment with nocodazole (left), after treatment with nocodazole (center), and after nocodazole treatment and washing (right). The two CEM cells shown for each treatment group were representative of the population of cells examined. Scale bar is 10 μm.

To determine whether the intracellular concentration of CD86 in L86SN-CEM cells was associated with the nucleolus, cells were immunostained with the nucleolus-specific Ab AE-3 (Fig. 2A, left), anti-CD86 Ab (Fig. 2A, center), or both Abs simultaneously (Fig. 2A, right) and analyzed by ICM. Double immunostaining (Fig. 2A, right) clearly demonstrated that the intracellular reservoir of CD86 was not associated with the nucleolus. ICM analysis of L86SN-CEM cells stained with anti-Golgi 58K protein Ab (Fig. 2B, left) showed a single focal concentration of fluorescence that was very similar in appearance and localization to that observed for intracellular CD86 staining (Fig. 2B, center), and double staining with both Abs (Fig. 2B, right) suggested possible Golgi localization of the intracellular CD86 protein. To examine this possibility further, L86SN-CEM cells were treated with BFA to disorganize the Golgi apparatus, and then immunostained and analyzed by ICM. The normal staining pattern observed on untreated L86SN-CEM cells with anti-Golgi 58K protein Ab (Fig. 2C, left) was no longer evident after BFA treatment (Fig. 2C, center), and reappeared as the Golgi apparatus reassembled when BFA was washed from the culture (Fig. 2C, right). No observable change in the pattern of CD86 localization in L86SN-CEM cells (Fig. 2D, left) was observed after BFA treatment (Fig. 2D, center), or after BFA was washed from the cells to reassemble the Golgi apparatus (Fig. 2D, right).

To examine the relationship between the microtubular network and the intracellular concentration of CD86 in L86SN-CEM cells, a series of experiments was performed using nocodazole. Anti-β-tubulin Ab was used to immunostain the cellular microtubules for analysis by ICM. The normal staining pattern observed on untreated L86SN-CEM cells with anti-β-tubulin Ab (Fig. 2E, left) was no longer evident after nocodazole treatment (Fig. 2E, center), and reappeared as the microtubules reassembled when nocodazole was washed from the culture (Fig. 2E, right). The intracellular focal concentration of CD86 in L86SN-CEM cells (Fig. 2F, left) was also disorganized after nocodazole treatment (Fig. 2F, center), although cell surface CD86 localization was still apparent. When nocodazole was washed from the culture, the intracellular focal concentration of CD86 reappeared (Fig. 2F, right). These results were reproduced in six independent experiments with three different L86SN-CEM cell clones.

Cell surface localization of CD80 and CD86 in unstimulated and OKT3-stimulated peripheral blood T lymphocytes does not involve an intracellular concentration of either protein

Localization of CD80 and CD86 in peripheral blood T lymphocytes was performed by FACS and ICM analysis on unstimulated and OKT3-stimulated cells. Unstimulated T lymphocytes did not express detectable cell surface CD80 or CD86 by FACS analysis (not shown), and there was no evidence of an intracellular focal concentration of either protein by ICM (Fig. 3, A and D, respectively). After OKT3 stimulation, the T lymphocytes were examined daily, and at day 8, cell surface CD80 and CD86 was first detected by FACS (not shown) and ICM, peaked in intensity by day 10 (Fig. 3, B and E, respectively), and began to decline again by day 14 (Fig. 3, C and F, respectively). At no time during this 14-day period was an intracellular focal concentration of either protein observed in a total of seven independent experiments. Isotype-control Ab staining was negative at each time point by FACS analysis and ICM (not shown).

FIGURE 2. Characterization of the intracellular focus of CD86 protein in L86SN-transduced CEM cells. Shown are ICM analyses of (A) L86SN-CEM cells immunostained with nucleolus-specific Ab AE-3 (left), anti-CD86 Ab (center), or both Abs simultaneously (right); (B) L86SN-CEM cells immunostained with Golgi-specific Ab (left), anti-CD86 Ab (center), or both simultaneously (right); (C) L86SN-CEM cells immunostained with Golgi-specific Ab before treatment with BFA (left), after treatment with BFA (center), and after BFA treatment and washing (right); (D) L86SN-CEM cells stained with anti-CD86 Ab before treatment with BFA (left), after treatment with BFA (center), and after BFA treatment and washing (right); (E) L86SN-CEM cells stained with anti-β-tubulin Ab before treatment with nocodazole (left), after treatment with nocodazole (center), and after nocodazole treatment and washing (right); and (F) L86SN-CEM cells stained with anti-CD86 Ab before treatment with nocodazole (left), after treatment with nocodazole (center), and after nocodazole treatment and washing (right). The two CEM cells shown for each treatment group were representative of the population of cells examined. Scale bar is 10 μm.

FIGURE 3. Cell surface localization of endogenous CD80 and CD86 in stimulated peripheral blood T lymphocytes. Shown are ICM analyses of endogenous CD80 expression by (A) unstimulated T lymphocytes, (B) T lymphocytes 10 days after OKT3 stimulation, and (C) T lymphocytes 14 days after OKT3 stimulation. ICM analysis of endogenous CD86 expression by (D) unstimulated T lymphocytes, (E) T lymphocytes 10 days after OKT3 stimulation, and (F) T lymphocytes 14 days after OKT3 stimulation. Isotype-control Ab staining was negative (not shown). Scale bar is 10 μm.
Endogenous CD80 and CD86 expression by B lymphocytes does not involve intracellular concentration of either protein

Peripheral blood B lymphocytes were isolated from the purified PBMC fraction by depletion of T lymphocytes and monocytes, and the B lymphocytes identified and characterized by FACS analysis. As expected, CD80 and CD86 were not detectable on the surface of unstimulated B lymphocytes by FACS analysis. After 24 h of stimulation by in vitro culture, both CD80 and CD86 were detectable by FACS analysis and ICM, and the cells remained positive for at least 48 h in vitro (not shown). At no time during the 48 h in vitro culture of peripheral blood B lymphocytes was an intracellular focal concentration of CD80 or CD86 protein observed by ICM (not shown).

Induction of CD80 expression in peripheral blood monocytes involves cell surface localization, but no intracellular concentration of the protein

Freshly isolated monocytes had no detectable cell surface CD80 by FACS analysis (Fig. 4A, red line, MFI 8), and there was no evidence of an intracellular concentration of CD80 (Fig. 4B) detected by ICM. After 24 h of stimulation by in vitro culture, approximately 70% of the monocyte population demonstrated cell surface CD80 localization by FACS (Fig. 4A, green line, MFI 103), but no intracellular concentration of protein was observed by ICM (Fig. 4C). After 48 h of stimulation by in vitro culture, more than 90% of the monocytes demonstrated surface localization of CD80 (Fig. 4A, blue line, MFI 156) by FACS analysis, but no intracellular concentration of the protein was observed by ICM (Fig. 4D). Iso-type-control Ab staining was negative by FACS and ICM (not shown). The results were reproduced in three independent experiments with monocytes isolated from different PBMC donors.

Monocytes constitutively express CD86 and have an intracellular reservoir of the protein that becomes depleted as cell surface expression increases

Although there was some variation among the PBMC donors used in the study, between 60 and 80% of the purified peripheral blood monocytes consistently and constitutively expressed CD86 immediately after isolation, and cell surface fluorescence staining was clearly evident by FACS analysis (Fig. 4E, red line, MFI 67) and ICM (Fig. 4F). ICM also revealed an intracellular focal concentration of CD86 in the monocytes (Fig. 4F) that was similar in appearance to that observed in L86SN-CEM cells (Fig. 1B). After 24 h of stimulation by in vitro culture, the surface CD86 fluorescence intensity had increased (Fig. 4E, green line, MFI 177) and the intracellular CD86 focal concentration had disappeared from approximately 50% of the monocyte population (Fig. 4G). After 48 h of stimulation by in vitro culture, more than 90% of the purified monocytes were positive for cell surface CD86 by FACS analysis (Fig. 4E, blue line, MFI 268), and the intracellular concentration of CD86 was only detectable in less than 5% of the population (Fig. 4H). This relationship between increasing cell surface localization of CD86 and decreasing intracellular concentration of the protein as a function of time in vitro was reproduced in three independent experiments with monocytes from different PBMC donors. Percentages were derived by counting more than 150 cells in each ICM field.

The intracellular reservoir of CD86 protein in peripheral blood monocytes is not due to concentration within the Golgi complex, and requires a network of intact microtubules to retain structural integrity

A series of BFA and nocodazole studies was performed on peripheral blood monocytes from normal donors to characterize the intracellular reservoir of CD86 protein. To examine any relationship between the reservoir and the Golgi, freshly isolated monocytes were treated with BFA to disorganize the Golgi apparatus, and then immunostained and analyzed by ICM. The ICM staining pattern observed when untreated monocytes were incubated with an anti-Golgi 58K protein Ab (Fig. 5A, left) was no longer evident after BFA treatment (Fig. 5A, center), and reappeared as the Golgi apparatus reassembled when BFA was washed from the culture (Fig. 5A, right). In contrast, there was no observable change in the intracellular concentration of CD86 in monocytes incubated with anti-CD86 Ab (Fig. 5B, left) after BFA treatment (Fig. 5B, center),
or when BFA was washed from the culture to reassemble the Golgi apparatus (Fig. 5B, right).

To examine any relationship between the microtubular network and the intracellular reservoir, a series of experiments was performed using nocodazole. Anti-β-tubulin-Ab was used to immunostain the cellular microtubules for analysis by ICM. The staining pattern observed when freshly isolated monocytes were incubated with anti-β-tubulin Ab (Fig. 5C, left) was no longer evident after nocodazole treatment (Fig. 5C, center), but did reappear as the microtubules reassembled when nocodazole was washed from the culture (Fig. 5C, right). Similarly, the intracellular reservoir of CD86 in freshly isolated monocytes incubated with anti-CD86 Ab (Fig. 5D, left) was also disorganized after nocodazole treatment (Fig. 5D, center), and reappeared rapidly when nocodazole was washed from the culture (Fig. 5D, right). These results were reproduced in three independent experiments with monocytes isolated from different PBMC donors.

The type I integral membrane proteins CD11b and CD4 do not colocalize with intracellular CD86 in peripheral blood monocytes

The ICM pattern of intracellular CD86 concentration in peripheral blood monocytes incubated with anti-CD86 Ab (Fig. 6A) was distinctly different from that obtained when monocytes were incubated with anti-CD11b Ab and examined by ICM (Fig. 6B). The CD11b protein was found to be predominantly associated with the cell surface membrane of freshly isolated monocytes, with some punctate intracellular fluorescence activity also evident (Fig. 6B). This punctate pattern of CD11b localization, however, was entirely different from that observed for intracellular CD86 (Fig. 6A). When incubated with anti-CD4 Ab, freshly isolated monocytes were only weakly positive for cell surface CD4 by FACS analysis, and no intracellular concentration of CD4 protein was detected by ICM (not shown). As a control for CD4 immunostaining, L86SN-CEM cells were incubated with anti-CD4 Ab, and examined by ICM. Again, a punctate intracellular staining pattern was observed (Fig. 6C) that differed significantly from the typical intracellular CD86 localization pattern (Fig. 6A).

Discussion

We are developing an immunotherapy strategy for the treatment of pediatric cancer based on genetically modifying tumor cells to express the costimulatory molecules CD80 and CD86. Our initial studies, using retrovirus-mediated transduction of human lymphoid (CEM) and nonlymphoid (HeLa) tumor cell lines, were designed to evaluate the stability of exogenous CD80 and CD86 gene expression, the cellular processing of the gene products, and the efficacy of costimulatory function in in vitro lymphoproliferation assays. On commencing the gene transfer studies, however, we were intrigued to observe that retrovirus-mediated expression of CD80 and CD86 in T lymphoblastoid CEM cells resulted in an unusual intracellular focal concentration of both proteins. Interestingly, this intracellular focal concentration of protein was never observed in any of the L80SN-HeLa or L86SN-HeLa clones examined.
A series of experiments was performed on L86SN-CEM cells to determine the nature of the intracellular concentration of CD86 protein. We eliminated the possibility of an interaction with the CEM cell nucleus by colocalization studies with anti-CD86 and a nucleus-specific Ab. We then addressed the possibility that the intracellular concentration of CD86 was due to storage within the Golgi complex. ICM localization studies were performed on L86SN-CEM cells before and after treatment with BFA to disrupt the Golgi apparatus, and revealed that the intracellular concentration of CD86 was not due to storage within the Golgi. The relationship between the intracellular concentration of CD86 and the integrity of the microtubules was then examined by treating L86SN-CEM cells with nocodazole, a compound that disrupts microtubule organization. The intracellular focal concentration of CD86 in L86SN-CEM cells was disrupted by nocodazole treatment, and rapidly restored after the compound was washed from the cultures. Evidently, intact microtubules are required to retain the structural integrity of the intracellular focal concentration of CD86 in transduced CEM cells.

Although the results of these initial studies were interesting, they were based on a genetically modified T lymphoblastoid cell line. To ensure that the observations were not peculiar to CEM cells, we extended our studies to an analysis of endogenous CD80 and CD86 expression in PBL and peripheral blood monocytes. Consistent with previous reports, FACS analyses revealed that 1) only stimulated T lymphocytes, B lymphocytes, and monocytes expressed CD80; 2) only stimulated T and B lymphocytes expressed CD86; and 3) both unstimulated and stimulated monocytes expressed CD86 (2, 6, 7, 9–12). We then examined purified PBL and peripheral blood monocytes by ICM for any intracellular concentration of CD80 or CD86 protein. The ICM experiments revealed that only monocytes, which are known to constitutively express CD86, had an intracellular focal concentration of CD86 protein. Moreover, the intracellular localization of CD86 in peripheral blood monocytes was very similar in appearance to that observed in the L86SN-CEM cells. The observation of an intracellular focal concentration of CD86 in monocytes, and the absence of a similar concentration of CD80 in these cells, suggested that constitutive gene expression was important in the process. This hypothesis would explain why no intracellular concentration of CD80 or CD86 was ever observed in T or B lymphocytes, and was consistent with the CEM cell experiments in which both genes were constitutively expressed from the retrovirus promoter. The absence of an intracellular concentration of CD80 or CD86 in the transduced HeLa cells, however, may indicate that the process is restricted to certain cell types. Further studies will be required to address this question.

The monocyte expression studies also revealed an interesting temporal relationship between the intracellular concentration of CD86 and the surface membrane localization of the protein. As the peripheral blood monocytes were stimulated by in vitro culture, both the absolute number of cells positive for surface membrane CD86 and the mean fluorescent intensity of the population increased as a function of time. Simultaneously, the intracellular concentration of CD86 protein decreased. The most likely explanation for this observation was that CD86 was being transported from an intracellular reservoir to the monocyte surface membrane in response to in vitro stimulation. In an attempt to further explore this antegrade CD86 transport hypothesis, a series of experiments was performed on peripheral blood monocytes to determine the nature of the intracellular reservoir. Similar to experiments described for the L86SN-CEM cells, BFA and nocodazole were used to treat freshly isolated peripheral blood monocytes to investigate any relationships between the intracellular CD86 reservoir, the Golgi, and the microtubules. Consistent with the earlier findings, BFA treatment revealed that the intracellular reservoir of CD86 in peripheral blood monocytes was not due to storage within the Golgi. In addition, nocodazole treatment revealed that intact microtubules were necessary for the intracellular reservoir of CD86 to retain structural integrity in peripheral blood monocytes. Both of these results further support a model in which the intracellular concentration of CD86 represents a functional reservoir of translated protein available for rapid and sustained deployment to the monocyte cell surface membrane in response to stimulation.

As mentioned earlier, CD86 is a type I integral membrane glycoprotein of the Ig supergene family. A question raised by our observations was whether or not all constitutively expressed integral membrane proteins of this family would be concentrated within the intracellular reservoir in monocytes. To address this question, we performed FACS analyses and ICM localization studies on peripheral blood monocytes with Ab to CD4 and CD11b. CD4 is a type I integral membrane protein of the Ig supergene family constitutively expressed by monocytes, and CD11b is a type I integral membrane protein of the integrin family constitutively expressed by approximately 80% of monocytes. ICM analysis revealed no intracellular concentration of CD4 in peripheral blood monocytes, and a punctate pattern of intracellular CD11b that was entirely different from the typical focal concentration observed with CD86. Clearly, not all constitutively expressed type I integral membrane proteins concentrate within the reservoir.

Collectively, the biologic significance of our observations may be related to the temporal regulation of lymphocyte costimulation. The interaction between CD86 on the surface of an APC, and CD28 (which is expressed on 95% of resting CD4+ T lymphocytes) is thought to be crucial for the initiation of a significant subset of T lymphocyte-mediated immune responses (6–10, 12). Since CD86 is constitutively expressed by monocytes, this costimulation and activation of T lymphocytes can occur immediately if an appropriate Ag is present. In contrast, the interaction between CD80 on an APC and CTLA-4 on a T lymphocyte, which is believed to be more important in the maintenance and/or modulation of the immune response, can only occur some hours after both cells have been stimulated to express these counterreceptors (21–23). Therefore, the presence of an intracellular reservoir of CD86 that could allow rapid and sustained transport of the protein to the monocyte surface membrane would seem to be biologically advantageous and entirely consistent with the current tenets of lymphocyte activation by costimulation.

Structurally, the intracellular reservoir described in this report probably represents a highly organized concentration of transport vesicles capable of rapid deployment of CD86 to the monocyte surface membrane. Although similarities exist between our results and a recent report describing intracellular storage and rapid release of Fas ligand from activated monocytes (24), the fluorescence localization patterns of CD86 and Fas ligand appear to be completely different. More striking parallels appear to occur between the CD86 reservoir in monocytes and the recently identified neuronal cell storage organelle, the botrysome. The botrysome is a discrete organelle located between the Golgi and the endoplasmic reticulum, and has been shown to be involved in intracellular protein trafficking in response to cellular activation (25). Similarities between the subcellular location and size of the neuronal cell botrysome (1–4 μm), and the intracellular reservoir for CD86 in monocytes are compelling, and future studies will attempt to examine any relationship that might exist. These studies, in addition to precise kinetic analyses of the trafficking and concentration of CD86 in monocytes, will be necessary to fully understand this...
novel reservoir, and may provide further insights into the temporal regulation of APC function in the cell-mediated immune response.

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