Soluble CD86 Is a Costimulatory Molecule for Human T Lymphocytes

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Summary

CD86 is an important costimulatory molecule for the priming and activation of naive and memory T cells, respectively. Here, we show that soluble CD86 is detected in human serum. Soluble CD86 is produced by resting monocytes and results from an alternatively spliced transcript (CD86ΔTM) characterized by deletion of the transmembrane domain. Recombinant CD86ΔTM binds to CD28 and CTLA-4 and induces the activation of T cells after stimulation with anti-CD3 mAb. CD86ΔTM also induces IFNγ production by virus-specific CD8+ memory human T cells stimulated with the Flu M1 peptide. The concentrations of soluble CD86 found in human serum are sufficient to induce biological activity. Soluble CD86 molecule, therefore, appears to be a functional costimulatory molecule playing a potentially important role in immune surveillance.

Introduction

Naive T cells require primary and costimulatory signals from antigen-presenting cells (APC) to be functionally activated. The primary antigen-specific signal initiates activation with costimulatory signals inducing clonal expansion and differentiation into effector cells (Lenschow et al., 1996; Chambers and Allison, 1997; Greenfield et al., 1998). Costimulatory signals are provided by the interaction of membrane molecules expressed by T cells and APC. The biological activity of the costimulatory molecule CD28 has been extensively studied. CD28 is constitutively expressed by T cells and interacts with the B7 molecules CD80 (B7.1) (Yokochi et al., 1982; Linsley et al., 1990) and CD86 (B7.2) (Azuma et al., 1993; Freeman et al. 1993). This interaction results in an increased T cell proliferation, IL-2 production, and resistance to apoptosis (Linsley et al., 1991a; Croft et al., 1992). T cells that bind antigen and do not receive a costimulatory signal are thought to die or to become anergic (Harding et al., 1992; Gribben et al., 1995). More recently, ICOS, a new member of the CD28 family, has been described (Hutloff et al., 1999). The expression of ICOS on naive T cells is induced by activation (Hutloff et al., 1999; Yoshinaga et al., 1999). ICOS interacts with B7-RP1 (Yoshinaga et al., 1999), also described as B7h (Swallow et al., 1999), which is constitutively expressed on B cells and macrophages (Yoshinaga et al., 1999) and upregulated by activation (Swallow et al., 1999). B7-H1, a third member of the B7 family, is a costimulatory molecule for human T cells, the ligand for which remains undetermined (Dong et al., 1999). In addition to CD28 and ICOS, activated T cells may also transiently express CTLA-4, a second ligand for CD80 and CD86 (Linsley et al., 1991b; Azuma et al., 1993). CTLA-4 engagement can transduce an "off" signal, disengaging T cells from further activation and proliferation (Walunas et al., 1994; Krummel and Allison, 1995; Saito, 1998). However, only CD28 is constitutively expressed by resting naive T cells, suggesting that the CD28/B7 ligand pairs play an important role in the generation of an immune response. In agreement with this observation, CD28-deficient mice (Shahinian et al., 1993) and transgenic mice producing a soluble CTLA-4-Fc molecule (Linsley et al., 1992) present a reduced Th response.

CD80 and CD86 are type I membrane glycoproteins belonging to the immunoglobulin supergene family (Bajorath et al., 1994). In humans, their expression patterns differ according to the nature of the APC. CD86 expression is constitutive on monocytes (Azuma et al., 1993; Hathcock et al., 1994) and dendritic cells (Caux et al., 1994; Yokozeki et al., 1996) and is upregulated by activation (Engel et al., 1994; McLellan et al., 1995). In contrast, CD80 is expressed at low levels on APC and upregulated following activation (Fleischer et al., 1996). CD80 and CD86 are also induced on T cells after activation (Hakamada-Taguchi et al., 1998; Jeannin et al., 1999). Despite having the same ligands, CD80 and CD86 appear to be involved in different mechanisms; CD80 can be more potent than CD86 in inducing an anti-tumoral response (Matulonis et al., 1996), while CD86 preferentially induces the production of a Th2 response (Freeman et al., 1995; Kuchroo et al., 1995). In CTLA-4-Ig transgenic mice, blocking the interaction of CD80/CD86 with their ligands results in defective T cell priming (Linsley et al., 1992). Due to its constitutive expression on human APC, CD86 has been suspected of being involved in the initiation of the immune response (Caux et al., 1994; Yi-qun et al., 1996; Manickasingham et al., 1998).

In this study, we report that soluble CD86 is detected in human serum. Soluble CD86 is produced by resting monocytes and results from an alternatively spliced transcript characterized by the deletion of the transmembrane domain. Soluble CD86 provides a costimulatory signal to memory human T cells.

Results

Detection of Soluble CD86 in Human Serum and Identification of a CD86 mRNA Spliced Variant

A soluble immunoreactive form of CD86 was detected in the freshly isolated serum of 10 of 60 healthy subjects
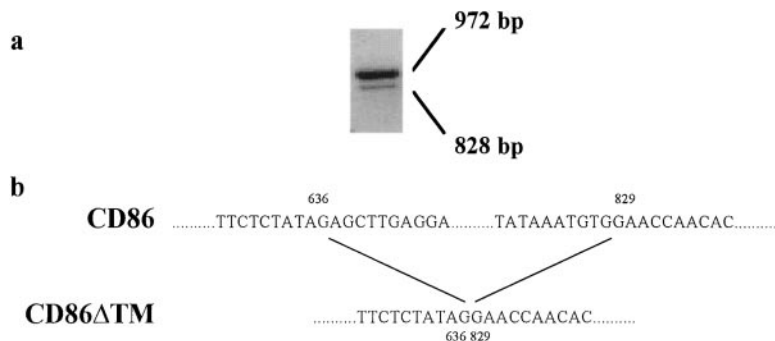
Figure 1. PBMC Express an Alternatively Spliced Variant of Human CD86 mRNA

(A) Identification of two CD86 transcripts in human PBMC. RT-PCR was performed to amplify the coding sequence of CD86 mRNA in human PBMC. The membrane CD86 and CD86ΔTM cDNA fragments were visualized by ethidium bromide staining.

(B) Nucleotide sequence at the splice junction of CD86 and CD86ΔTM. Nucleotide sequence at the splice junction and the deduced amino acid sequence of CD86ΔTM are shown.

(6.3 ± 4.5 ng/ml, mean ± SEM, ranging from 1 to 15 ng/ml), 6/40 cancer patients (3.7 ± 3.1 ng/ml, ranging from 1 to 10 ng/ml), and 8/40 subjects suffering from autoimmune disease (4.6 ± 2.1 ng/ml, ranging from 2 to 12 ng/ml) (data not shown). Soluble CD86 can be produced either by the shedding of the membrane form or through an alternative mRNA splicing. RT-PCR analysis of CD86 mRNA revealed the constitutive expression of two transcripts in resting human peripheral blood mononuclear cells (PBMC) (Figure 1A). The largest form (972 bp) was identical to the published CD86 sequence (Azuma et al., 1993; Freeman et al., 1993; data not shown). The smallest cDNA fragment (828 bp), named CD86ΔTM, has a deletion from nucleotide (nt) 686 (starting from ATG codon) to nt 829 and encodes for a 275 amino acid polypeptide. According to the *cd86* genomic organization (Jellis et al., 1995), this cDNA is generated by alternative splicing of the CD86 mRNA in which the exon 6 (encoding for the transmembrane region) is deleted (Figure 1B). The deletion does not affect the reading frame (Figure 1B).

Our results show that a soluble form of CD86 can be detected in human serum and that human PBMC express two CD86 mRNA variants.

Recombinant CD86ΔTM Is Expressed as a Soluble Molecule

The deletion of exon 6 (encoding for the transmembrane domain) suggested that CD86ΔTM could be produced as a soluble molecule. Recombinant CD86 and CD86ΔTM expressed in COS cells have an apparent molecular weight of 65 and 48 kDa in nonreducing conditions, respectively (Figure 2A). Membrane CD86 was detected by FACS on the surface of COS cells transfected with CD86 but not with CD86ΔTM cDNA (Figure 2B). A soluble molecule reactive with anti-CD86 mAbs was detected by ELISA in the cell-free supernatant of CD86ΔTM-transfected cells but not in the supernatants of CD86-transfected cells and mock cells (Figure 2C).

These results show that recombinant CD86ΔTM is a soluble molecule.

Soluble CD86 Detected in Human Serum Is Encoded by the CD86ΔTM Transcript

In order to selectively detect CD86ΔTM, a specific polyclonal serum was produced by immunizing rabbits with the peptide DEAQRVFKSSKTSSCDKSD, located within the intracellular domain of CD86. Western blotting analysis showed that the serum recognized both membrane CD86 and CD86ΔTM molecules produced in COS cells (Figure 3A). In ELISA, the polyclonal antibody recognized recombinant CD86ΔTM but not the CD86-Fc and CD80-

Fc protein (extracellular domain of CD86 or CD80 fused to the CH2 and CH3 domains of mouse Fcγ1) (Figure 3B). The preimmune rabbit serum did not recognize human
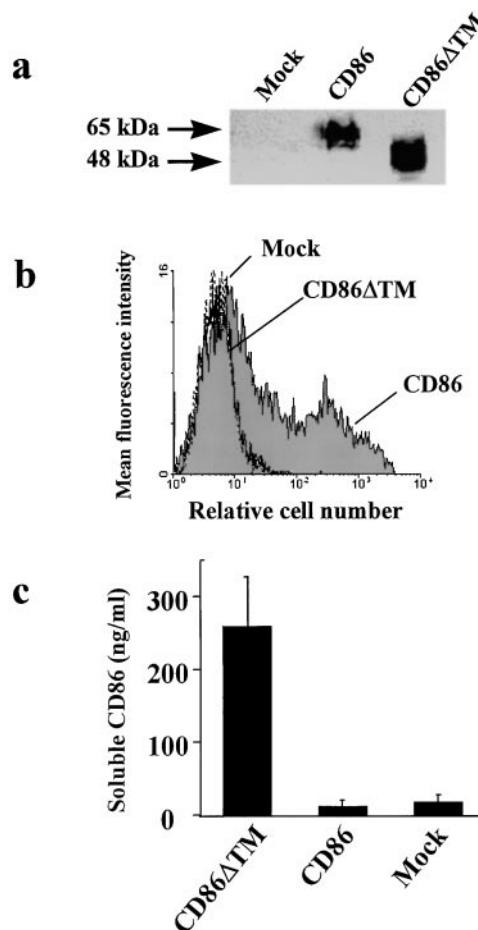

Figure 2. Recombinant CD86ΔTM Is Produced as a Soluble Molecule

COS cells were transiently transfected with the pCDNA3.1 vector containing CD86 or CD86ΔTM cDNA. After 48 hr, protein extracts were size separated by nonreducing SDS-PAGE, and CD86 expression was analyzed by Western blotting (A). Membrane CD86 expression was analyzed by FACS (B), and soluble CD86 was determined by ELISA in the concentrated culture supernatants (C). The concentration of soluble CD86 was determined using purified recombinant CD86ΔTM as a standard. Results are expressed in nanograms/milliliter (mean ± SD, n = 5).
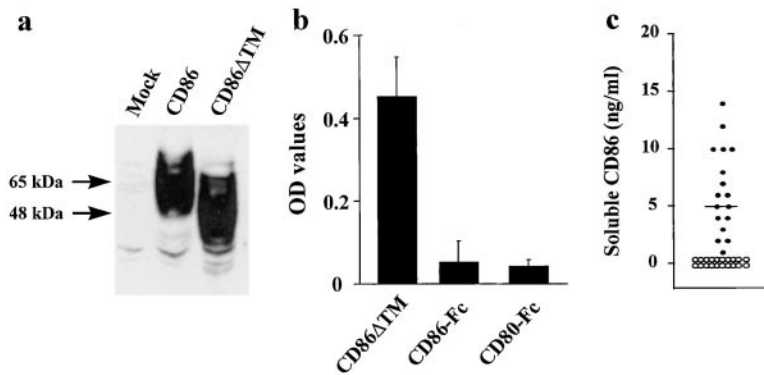
Figure 3. Soluble CD86 Detected in Human Serum Is Identical to CD86ΔTM

(A and B) Characterization of the anti-CD86ΔTM–specific polyclonal Ab. (A) Western blot analysis shows that the anti-CD86ΔTM polyclonal Ab recognizes both recombinant CD86ΔTM and membrane CD86 expressed by COS-transfected cells. (B) The anti-CD86ΔTM polyclonal Ab recognizes recombinant CD86ΔTM but not CD86-Fc and CD80-Fc–fusion proteins by ELISA. Results are expressed in optical density values.

(C) Soluble CD86ΔTM is detected in human serum. Serum containing the highest levels of soluble CD86 (n = 17) (closed circle) as determined by ELISA using the anti-CD86 mAb and negative serum (n = 20) (open circle) were tested by ELISA using the anti-CD86ΔTM polyclonal Ab. The concentration of soluble CD86 was determined using purified recombinant CD86ΔTM. Results are expressed in nanograms per milliliter.

serum and recombinant CD86ΔTM and CD86-Fc (data not shown). The CD86ΔTM-specific polyclonal antibody detected soluble CD86 in 17/24 CD86$^+$ serums, whereas no signal was observed in 20/20 CD86$^-$ serums tested (Figure 3C). The levels detected in the different serums with the ELISA for soluble CD86 or specific for CD86ΔTM were identical. However, due to a lower sensitivity of the ELISA, levels of soluble CD86 lower than 5 ng/ml were not detected in this assay.

Taken together, these results show that the soluble form of CD86 detected in human serum can be generated by translation of the CD86ΔTM mRNA.

Resting Monocytes Constitutively Express CD86ΔTM mRNA and Produce Soluble CD86

The expression of CD86 versus CD86ΔTM mRNA was evaluated in different cell types by RT-PCR. Results show that the two splice variants are constitutively expressed by nonstimulated monocytes (Figure 4A), while only CD86 mRNA was expressed by peripheral blood and tonsillar B cells, in vitro–generated dendritic cells, alveolar macrophages (Figure 4A), and different transformed cell lines expressing membrane CD86 (data not shown). None of the CD86 transcripts were detected in T cells, NK cells, neutrophils, lung epithelial cells, or microvascular endothelial cells (data not shown). Activation of monocytes with 100 ng/ml LPS, 10 U/ml IFNγ, or 1 μg/ml anti-CD40 mAb resulted in the preferential expression of membrane CD86 mRNA (Figure 4B). Using either mAbs (data not shown) or the CD86ΔTM-specific serum (Figure 3C), results showed that monocytes spontaneously released soluble CD86. As expected, no soluble CD86 was observed in B cell, dendritic cell, or alveolar macrophage culture supernatants. Soluble CD86 production was abolished in monocytes by activation with LPS, anti-CD40 mAb, or IFNγ (Figure 4C).

These data demonstrate that nonstimulated human monocytes produce soluble CD86ΔTM and that activation inhibits this production.

CD86ΔTM Is a Costimulatory Molecule

We further analyzed the biological activity of soluble CD86. In a first set of experiments, we evaluated whether the molecule CD86ΔTM can bind to its ligands, CD28 and CTLA-4. The expression of CD28 and CTLA-4 by COS cells was analyzed by FACS (Figures 5A and 5C). Results show that 10 μg/ml recombinant CD86ΔTM binds to CD28-transfected cells (MFI = 72 ± 8, n = 3)

(Figure 5B) or CTLA-4-transfected cells (MFI = 49 ± 6) (Figure 5D) but not to mock-transfected cells (Figures 5B and 5D). This binding is inhibited by 10 μg/ml anti-CD86 mAb (83% ± 10% and 94% ± 8% inhibition on CD28 and CTLA-4 transfected cells, respectively; mean ± SD, n = 4) (Figure 5B) and by 5 μg/ml CTLA-4-Fc (68% ± 8% and 75% ± 6% inhibition, respectively) (data not shown) but not by an anti-CD80 mAb (data not shown). Recombinant CD86ΔTM (10 μg/ml) also binds to the human T lymphoma cell line Jurkat (MFI = 38 ± 8, mean ± SD, n = 3) (data not shown), which constitutively expresses CD28. This binding is also inhibited by an anti-CD86 mAb (70% ± 12% inhibition) and CTLA-4-Fc (68% ± 8% inhibition) but not by an anti-CD80 mAb. We next evaluated the biological activity of recombinant soluble CD86ΔTM. In vitro assays showed that CD86ΔTM increased the proliferation of T cells stimulated with a suboptimal concentration of an anti-CD3 mAb. This effect was significant at 10 ng/ml (stimulation index [SI] = 8.4 ± 1.3; mean ± SD, n = 6; p < 0.05) and maximal at the highest concentration tested (1 μg/ml) (SI = 25 ± 4; p < 10^{-4}) compared to stimulation with the anti-CD3 mAb alone (SI = 2.5 ± 0.6) (Figure 6A). T cell proliferation was inhibited by 10 μg/ml neutralizing anti-CD86 mAb (80% ± 6% inhibition) or 5 μg/ml CTLA-4-Fc (82% ± 9% inhibition) but not by anti-CD80 and the isotype control mAbs (Figure 6B). No proliferation was induced by soluble CD86ΔTM in the absence of the anti-CD3 mAb. As a control, anti-CD3 plus anti-CD28 mAb induced a stronger T cell proliferation (SI = 74 ± 11; mean ± SD, n = 5) (Figures 6A and 6B). T cell proliferation was associated with cytokine production. CD86ΔTM, used at the optimal concentration of 100 ng/ml, induced a higher production of IFNγ (12 ± 2.5 ng/ml; mean ± SD, n = 6) (Figure 6C) and IL-2 (5.6 ± 0.9 ng/ml) (Figure 6D) by anti-CD3 mAb-stimulated T cells compared with a stimulation with the anti-CD3 mAb alone (4 ± 0.8 ng/ml and 1.9 ± 0.2 ng/ml , respectively) (Figures 6C and 6D). Both IFNγ and IL-2 production was inhibited by 10 μg/ml neutralizing anti-CD86 mAb (66% ± 10% and 52% ± 8% inhibition, respectively) or 5 μg/ml CTLA-4-Fc (72% ± 13% and 61% ± 9% inhibition, respectively) (Figures 6C and 6D) but not by anti-CD80 or isotype-matched mAbs (data not shown). As a control, IFNγ and IL-2 production by T cells activated with anti-CD3 plus anti-CD28 mAb was 25 ± 4.5 and 6.8 ± 1.1 ng/ml, respectively (Figures 6C and 6D). Lymphokine production was undetectable when soluble CD86ΔTM was used alone (data not shown). Previous studies have
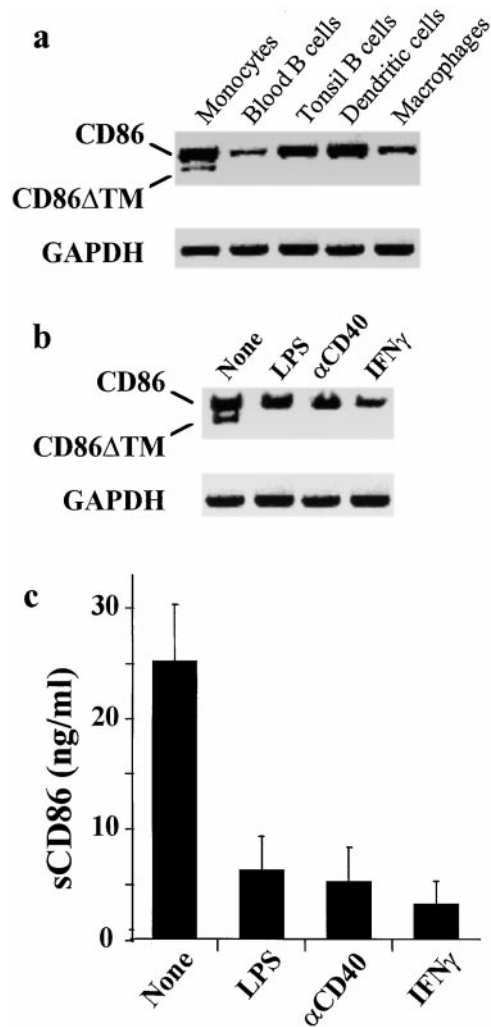
Figure 5. CD86ΔTM Binds to CD28 and CTLA-4

The binding of CD86ΔTM to its ligands CD28 and CTLA-4 was evaluated by FACS using CD28⁻ (A) and CTLA-4–transfected COS cells (C). The binding of c-myc–tagged CD86ΔTM on transfected cells (B and D) was evaluated using an anti-c-myc mAb (clone 9E10) revealed with an FITC-labeled anti-mouse Ig Ab. In some experiments, CD86ΔTM was incubated with neutralizing anti-CD86 mAb before the binding assay.

These results show that as previously reported for membrane CD86 (Linsley et al., 1991a; Azuma et al., 1993; Freeman et al., 1993), CD86ΔTM in association with anti-CD3 mAb potentiates proliferation and lymphokine production by T cells. CD86ΔTM acts, at least in part, by inducing the phosphorylation of Vav.

CD86ΔTM Costimulates Memory T Lymphocytes

Naive T cells require a strong costimulatory signal to be efficiently activated, while memory T cells require low stringency activation signals (Croft et al., 1992; Liu and Janeway, 1992; Yi-qun et al., 1996; Manickasingham et al., 1998). We have thus evaluated whether soluble CD86ΔTM can provide a similar activation signal to naive and memory T lymphocytes. Cell cycle analysis revealed that $16\% \pm 1.6\%$ (mean $\pm$ SD, n = 3) of CD45RO⁺ proliferate in response to CD86ΔTM plus anti-CD3 mAb, while only $11.2\% \pm 1.2\%$ ($p < 0.05$) proliferate in response to anti-CD3 mAb alone (Figure 7A). Naive T cells also proliferate in response to CD86ΔTM plus anti-CD3 mAb when compared to a stimulation with anti-CD3 mAb alone ($6.5\% \pm 0.6\%$ compared to $4.7\% \pm 0.9\%$, respectively) (Figure 7A). We have thus evaluated whether soluble CD86 provides the costimulatory signal required to activate antigen-specific memory cells. The influenza-specific memory CD8⁺ T cells circulate in a state whereby they can rapidly display effector function following stimulation (Bednarek et al., 1991; Lalvani et al., 1997). T cells from HLA-A2 subjects were stimulated for 6 hr with the influenza nonapeptide GILGFVFTL (Bednarek et al., 1991) plus CD86ΔTM, and intracytoplasmic IFNγ production was detected among CD8⁺ T cells. Representative data obtained from one of five subjects are presented. CD86ΔTM induced an increase of IFNγ production in CD45RO⁺ T cells compared with CD45RA⁺ T cells

Figure 4. CD86ΔTM Expression Is Restricted to Human Monocytes and Regulated by Activation

(A) CD86ΔTM mRNA is selectively expressed in monocytes. The expression of the CD86ΔTM trancript was analyzed by RT-PCR in freshly isolated monocytes, peripheral blood, tonsillar B cells, in vitro–generated dendritic cells, and alveolar macrophages. The amplified fragments were size separated on a 1% agarose gel and visualized by ethidium bromide staining.

(B) Regulation of CD86ΔTM mRNA expression in human monocytes. Human monocytes were either nonstimulated or stimulated with LPS, anti-CD40 mAb, or IFNγ. The CD86ΔTM transcript expression was analyzed after a 6 hr stimulation. RNA integrity and cDNA synthesis was verified by amplifying GAPDH cDNA.

(C) Nonactivated human monocytes produce soluble CD86ΔTM. Human monocytes were nonstimulated or stimulated with LPS, anti-CD40 mAb, or IFNγ. After an overnight incubation, soluble CD86 was detected by ELISA using the specific CD86ΔTM polyclonal serum as detection Ab. The concentration of soluble CD86 was determined using purified recombinant CD86ΔTM. Results are expressed in nanograms per milliliter.

reported that Vav is phosphorylated in response to CD28 ligation (Klasen et al., 1998). We thus evaluated whether CD86ΔTM could also induce the phosphorylation of this protein. Immunoprecipitation and Western blotting analysis showed that like the anti-CD28 mAb, cross-linked CD86ΔTM could induce the phosphorylation of Vav in Jurkat cells (Figure 6E).
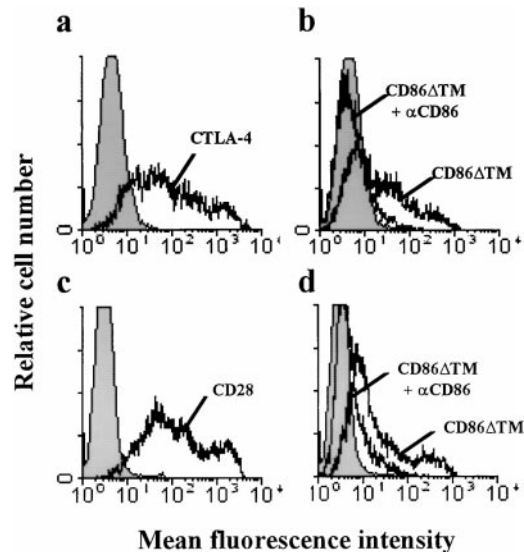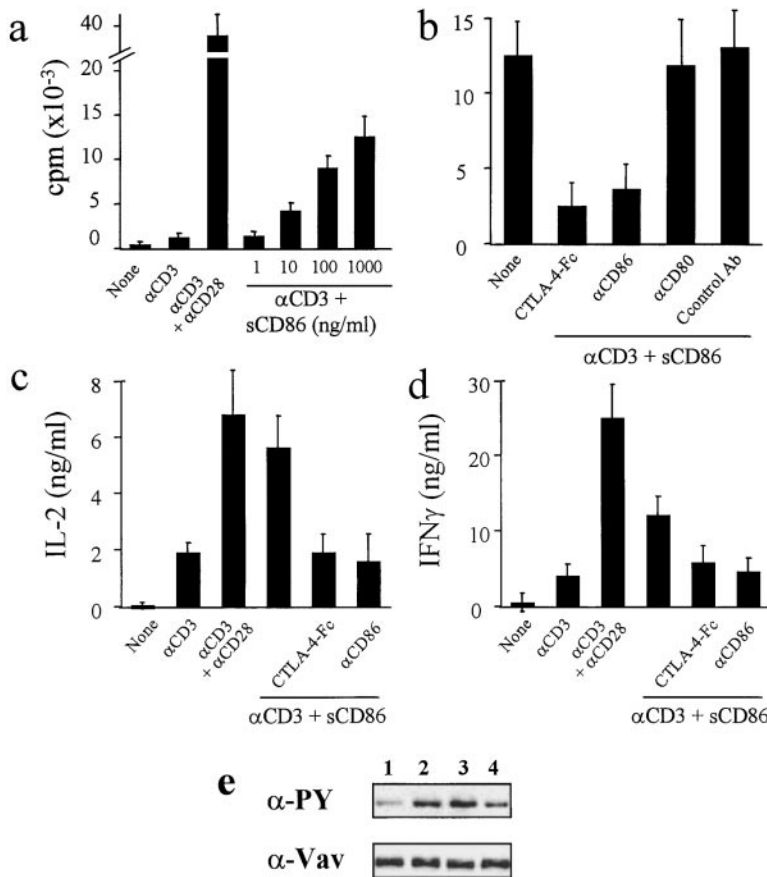
Figure 6. CD86ΔTM Is a Costimulatory Molecule for Human T Cells

(A and B) T cell proliferation was determined after 72 hr stimulation by [³H]thymidine incorporation. Results are expressed in counts per minute, mean ± SD. (A) CD86ΔTM potentiates the proliferation of anti-CD3 mAb–activated T cells. Human T cells were stimulated with a suboptimal concentration of anti-CD3 mAb without or with recombinant CD86ΔTM. Positive control of T cell proliferation was induced by anti-CD3 plus anti-CD28 mAbs. (B) CD86ΔTM-induced T cell proliferation is inhibited by blocking CD86. Human T cells were stimulated with a suboptimal concentration of anti-CD3 mAb plus CD86ΔTM without or with CTLA-4-Fc, anti-CD86, anti-CD80, or isotype control mAbs.

(C and D) CD86ΔTM potentiates the production of IFNγ and IL-2 by anti-CD3 mAb–activated T cells. T cells were stimulated with anti-CD3 mAb without or with recombinant CD86ΔTM. In some experiments, neutralizing anti-CD86 mAb or CTLA-4-Ig were added. IFNγ and IL-2 production was determined by FACS analysis as mentioned in the Experimental Procedures section. A stimulation with anti-CD3 plus anti-CD28 mAbs was performed as a control. Results are expressed in nanograms per milliliter.

(E) CD86ΔTM induces tyrosine phosphorylation of Vav. Jurkat T cells were nonstimulated (1) or stimulated with cross-linked CD86ΔTM (2), an anti-CD28 mAb (3), or with an anti-c-myc plus goat anti-Ig antibody (4) for 5 min at 37°C. After immunoprecipitation, the tyrosine phosphorylation of Vav was analyzed by immunoblotting using anti-phosphotyrosine mAb (α-PY, upper panel). The levels of Vav expression were determined by immunoblotting using an anti-Vav Ab (lower panel).

(0.66% and 0.03%, respectively) or nonstimulated cells (0.05% and 0.02%, respectively) (Figure 7B). Similar results were obtained by activating T cells with the peptide plus anti-CD28 mAb (1.4% and 0.06% among CD8⁺CD45RO⁺ and CD8⁺CD45RA⁺ cells produce IFNγ, respectively) (Figure 7B). In contrast, the frequency of IFNγ-producing cells was not modified when cells were stimulated with CD86ΔTM alone (Figure 7B).

These results show that CD86ΔTM costimulates antigen-specific memory T cells.

Discussion

We report here the detection of soluble CD86 in human serum. Soluble CD86 is generated from an alternative splicing of the CD86 mRNA (CD86ΔTM), which results in the deletion of the transmembrane domain. CD86ΔTM is expressed by nonactivated monocytes. Soluble recombinant CD86ΔTM potentiates proliferation and lymphokine production by human T cells stimulated with anti-CD3 mAb or a recall antigen.

The human *cd86* gene is a single-copy gene organized into eight exons (Jellis et al., 1995). The transcript CD86ΔTM is generated by the splicing of exon 6, which results in the deletion of the transmembrane domain. The acceptor and donor sites conform with the consensus splice-junction sequences (Mount, 1982). Alternatively spliced variants of murine B7 molecules have been

previously reported. A spliced variant of the CD80 mRNA, characterized by the deletion of exon 3 and resulting in the loss of the C-like domain, has been found in lymphoid organs (Inobe et al., 1994, 1996; Guo et al., 1995). Exons 1 and 5 of murine CD86 can be used in alternative fashion to generate four transcripts (differing in their 5′ untranslated and signal regions) that encode the same molecule (Borriello et al., 1995). An additional alternatively spliced variant of murine CD86 encoding a partial IgV-like domain has also been reported (Borriello et al., 1995). The expression of these spliced transcripts is controlled by activation, as observed here with human CD86. However, no spliced variant generating soluble CD86 has been described in mice, hindering the evaluation of biological activity of soluble CD86 in in vivo mouse models. Thus, to our knowledge, these data are the demonstration of a naturally occurring alternative splicing of the CD86 mRNA, resulting in the synthesis of a soluble costimulatory molecule. The Ig-like structure of CD86 is maintained by two intrachain disulfide bonds between Cys⁴⁰–Cys¹¹⁰ (V-type domain) and Cys¹⁵⁷–Cys²¹⁸ (C-type domain) (Azuma et al., 1993; Freeman et al., 1993; Bajorath et al., 1994) with ligand binding involving at least the V-type domain (Rennert et al., 1997). The reading frame of the CD86ΔTM mRNA is not affected. Moreover, recombinant CD86ΔTM binds to CD28 and CTLA-4 and costimulates T cells in a CD28-dependent manner, demonstrating that the binding sites of
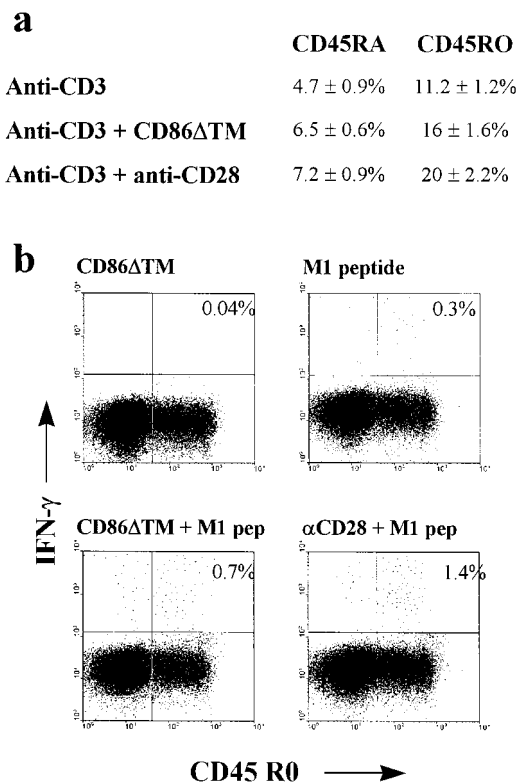
a

	CD45RA	CD45RO
Anti-CD3	4.7 ± 0.9%	11.2 ± 1.2%
Anti-CD3 + CD86ΔTM	6.5 ± 0.6%	16 ± 1.6%
Anti-CD3 + anti-CD28	7.2 ± 0.9%	20 ± 2.2%

b

Figure 7. CD86ΔTM Is a Costimulatory Molecule for Human Memory T Cells

(A) CD86ΔTM induces the proliferation of CD45RO$^+$ T cells. After stimulation with an anti-CD3 mAb, without or with CD86ΔTM, the phenotype of proliferating T cells was measured by Hoechst 33342 staining and labeling with anti-CD45RA and anti-CD45RO mAbs. T cell activation with anti-CD3 plus anti-CD28 mAb was performed as a control. Results are expressed as a percentage of cells in S+G2M phases of the cell cycle among naive and memory subpopulations, mean ± SD.

(B) CD86ΔTM induces IFNγ production by CD8$^+$ T cells stimulated with the influenza M1 peptide. PBMC from HLA-A2 donors were incubated with the flu M1 nonapeptide with or without CD86ΔTM. As a control, cells were incubated with the peptide plus an anti-CD28 mAb. The frequency of IFNγ-producing CD8$^+$ cells among the CD45RA$^+$ and CD45RO$^+$ T lymphocytes was determined by FACS. Representative results obtained with one subject are presented.

CD86ΔTM are conserved. These data are also in agreement with the observation that the V-like domain of B7 molecules is sufficient to activate T cells (Rennert et al., 1997).

As previously reported for membrane CD86 (Linsley et al., 1991a), recombinant soluble CD86ΔTM potentiates the proliferation and lymphokine production by anti-CD3 mAb–stimulated T cells. Interestingly, dimeric soluble CD86ΔTM (produced as a GST fusion protein) was equally potent as anti-CD28 mAb in increasing the proliferation of anti-CD3 mAb–stimulated T cells (data not shown). The covalent homodimeric molecules CD28 and CTLA-4 have two binding sites (2:2 stochiometry) (Linsley et al., 1995), and the oligomerization or clustering of membrane CD80/CD86 has been proposed in order to explain the higher avidity of CTLA-4-Ig to membrane CD86 compared to monomeric CD86 (Symington et al., 1993). This higher avidity should enhance the signaling by increasing the time of receptor occupancy by the ligand. These results are in agreement with the observation that anti-CD28 mAb (Linsley et al., 1991a) and dimeric CD86ΔTM transduce a more potent costimulatory signal than monomeric CD86ΔTM. The lower amplitude of T cell activation induced by monomeric CD86ΔTM could result either from a lower avidity between soluble CD86 and its ligand or a higher dissociation rate compared with membrane CD86, which could be responsible for the generation of a lower costimulatory signal. In contrast with monomeric CD86ΔTM, the cross-linking of the fusion protein CD86-Ig was required to potentiate the anti-CD3 mAb–stimulated T cell proliferation (Rennert et al., 1997). One can speculate that the monomeric structure of soluble CD86ΔTM is sufficient to activate T cells or that soluble CD86 can exist in a noncovalent oligomeric form. A recent study showing that soluble CD80 can form homodimers in solution (Ikemizu et al., 2000) is in favor of this hypothesis. Interestingly, cross-linked CD86ΔTM induced the phosphorylation of the CD28 signal-transducing molecule Vav (Klasen et al., 1998). Although we cannot exclude the possibility that CD86ΔTM may also interact with CTLA-4 and thus generate an inhibitory signal, these data demonstrate that soluble CD86 acts, at least in part, by inducing a CD28-dependent activation signal.

Membrane CD86, which is constitutively expressed by monocytes, B cells, and dendritic cells (Caux et al., 1994; Engel et al., 1994; Vyth-Dreese et al., 1995; Yokozeki et al., 1996), has been shown to play an important role in the priming of naive T cells and activation of memory T cells (Yi-qun et al., 1996; Manickasingham et al., 1998). Since the activation of naive T cells requires strong activation signals that are provided by professional APC (Liu and Janeway, 1992), and the activation of recently activated memory T cells can be elicited with anti-CD3 mAb alone (Van de Velde et al., 1993), most memory T cells are still dependent on CD28 triggering for their activation (Yi-qun et al., 1996). FACS analysis revealed that soluble CD86ΔTM potentiated the proliferation of memory and naive T cells.

We have also observed that CD86ΔTM potentiates the activation of memory T cells stimulated with a recall antigen: recombinant CD86ΔTM increased the frequency of IFNγ-producing influenza-specific memory T cells stimulated with the flu M1 peptide. These results are in agreement with the fact that memory T lymphocytes are less dependent than naive T cells on the amplitude of the costimulatory signal to be efficiently activated (reviewed by Croft, 1994). These data suggest that soluble CD86 could be involved (1) in the activation of memory T cells and/or (2) in maintaining the "activation" status of CD45RO$^+$ T lymphocytes (Zinkernagel et al., 1996). A similar role for membrane CD86 in the regulation of a memory antigen–specific restimulation has been previously reported (Keane-Myers et al., 1997). Moreover, the expression of CD86ΔTM mRNA and production of soluble CD86 are restricted to nonactivated human monocytes, suggesting that soluble CD86 could be involved in the early phase of the immune response, when activation of memory T cells may occur. These results are in favor of a role for soluble CD86 in the immune surveillance. In contrast, the initiation of a specific immune response requires the exclusive expression of membrane CD86, which provides a potent costimulatory signal to naive T cells.

In agreement with an important immunoregulatory role played by soluble CD86, an immunoreactive form

of CD86 was detected in human serum. The existence of a biologically active form of soluble CD86 has been previously suspected. Porcine endothelial cells constitutively release a soluble factor that mediates a CD28-dependent T cell proliferation (Davis et al., 1996). However, we did not detect an alternatively spliced variant of CD86 in human endothelial cells, suggesting that soluble CD86 found in human serum could be predominantly produced by monocytes. Interestingly, the concentration range of soluble CD86 found in human serum is similar to that which induces in vitro biological activity. We cannot exclude the possibility that in some conditions, soluble CD86 could also be generated by shedding of the membrane form. Collectively, our results show that serum-soluble CD86, generated by alternative splicing, is likely to be produced by circulating monocytes, which represent a large fraction of the PBMC. This soluble costimulatory molecule can thus play an important role in the immune surveillance against recall antigens and acts as a constant activation stimulus involved in the regulation of memory human T cell homeostasis.

Experimental Procedures

Detection of Soluble CD86

Soluble CD86 was evaluated by ELISA using commercial mAbs in the culture supernatants or serum of healthy subjects (n = 60) and subjects suffering from cancer (n = 40) and autoimmune disease (n = 40). In order to verify that soluble CD86 is identical to CD86ΔTM and not generated by shedding of the membrane form, a specific CD86ΔTM polyclonal antibody was generated by immunization of a rabbit with the peptide DEAQRVFKSSKTSSCDKSDT, whose sequence is localized in the intracytoplasmic tail of CD86. This domain is conserved in soluble CD86ΔTM. After incubation with the human serum, plates were incubated with the rabbit serum diluted at 1:5000, and bound Abs were detected using a peroxidase-labeled anti-rabbit Ig Ab (Pierce, Rockford, IL) revealed with the substrate o-phenylene diamine. Results are expressed in optical density values after subtraction of the value obtained with in the preimmune rabbit serum.

Isolation and Activation of Human Cells

Human PBMC were isolated by centrifugation on Ficoll/Paque (Amersham Pharmacia Biotech, Uppsala, Sweden). T cells were purified by rosetting with sheep red blood cells; the purity, determined by flow cytometry on a FACScan cytofluorometer (Becton Dickinson, San Jose, CA) using an FITC-labeled anti-CD3 mAb (Becton Dickinson), was >95%. Human peripheral blood B cells were purifed by flow cytometry using an FITC-labeled anti-CD19 mAb (Becton Dickinson). Tonsillar B cells were isolated from Ficoll-separated tonsillar mononuclear cells by a two-step negative-selection procedure using sheep erythrocyte rosetting and magnetic bead depletion (Dynal, Oslo, Denmark) to remove T cells, as previously reported (Jeannin et al., 1995). B cell purity was routinely >98% as determined by FACS analysis using an FITC-labeled anti-CD20 mAb (Becton Dickinson). Monocytes were either purified by FACS on the basis of FSC/SSC parameters or using immunomagnetic beads coated with an anti-CD14 mAb (MACS; Miltenyi, Bergish Gladbach, Germany); purity was >98%. Dendritic cells were generated in vitro by activating monocytes for 7 days with IL-4 plus GM-CSF (R&D Systems, Abingdon, UK); purity was >98% as assessed by CD1a expression and absence of CD83 labeling (Immunotech, Marseille, France). In some experiments, monocytes were cultured in complete medium (RPMI 1640 medium supplemented with 5% FCS and antibiotics reagent) (all from Life Technologies, Cergy Pontoise, France) and were either unstimulated or stimulated with 10 ng/ml LPS (Sigma, St Louis, MO), 10 U/ml IFNγ (R&D Systems), or 1 μg/ml anti-CD40 mAb (Ancell, Bayport, MN).

Cloning and Sequencing of CD86 mRNA Variants

Total RNA was extracted with the Trizol reagent (Life Technologies), and poly(A)$^+$ RNA was purified using oligo-dT-coated magnetic beads (Dynal) according to the manufacturer's recommendations. Single-strand cDNA was synthesized using 1 μg of poly(A)$^+$ RNA by reverse transcription using an oligo-dT primer (Amersham Pharmacia Biotech). Total RNA from human alveolar macrophages, lung epithelial cells, and microvascular endothelial cells were kindly provided by Dr. P. Gosset (Institut Pasteur de Lille, Lille, France). PCR reactions were performed with cDNA corresponding to 10 ng poly(A)$^+$ RNA. CD86 mRNA was amplified with primers designed to amplify the entire coding sequence of CD86 (5′-ATGGGACTGAGTA ACATTCTCTTTGTGATGGCCT-3′ and 5′-CTCGAGTTAAAAACATGT ATCACTTTTGTCGCATGA-3′). The PCR reaction was performed as follows: 94°C for 4 min, then 25 cycles 94°C for 30 min, 60°C for 30 min, and 72°C for 1 min followed by a final extension at 72°C for 5 min. The amplified fragments were size separated on a 1% agarose gel and visualized by ethidium bromide. After excision from the gel, each amplified cDNA fragment was subcloned into a TA cloning vector (pCRII; InVitrogen, Leek, The Netherlands). Sequencing was performed using the ABI-PRISM Dye Terminator Cycle Ready Reaction kit (Perkin Elmer, Foster City, CA). Two cDNAs of different size were amplified and called CD86 and CD86ΔTM (see the Results section). The expression of CD86 mRNA in activated versus nonstimulated human monocytes was evaluated by PCR using the above mentioned primers. RNA integrity and cDNA synthesis was verified by amplifying GAPDH cDNA (5′-TCCACCACCCTGTTGCT GTA-3′ and 5′-ACCACAGTCCATGCCATCAC-3′).

CD86 and CD86ΔTM Transfected COS Cells

The CD86 and CD86ΔTM cDNAs were subcloned in the vector pCDNA3.1 and pCDNA3.1-A Myc-His (InVitrogen) and used to transfect COS cells. COS cells (ATCC, Manassas, VA) were cultured in DMEM medium supplemented with 10% FCS, L-glutamine, and antibiotics (all from Life Technologies) and transfected by lipofection (Fugene; Boehringer Mannheim, Mannheim, Germany). Expression of membrane recombinant molecules was evaluated by FACS analysis using FITC-labeled anti-CD86 mAb (PharMingen, San Diego, CA). The production of soluble recombinant CD86 was assessed by ELISA in the cell-free supernatants. Briefly, the anti-CD86 mAb (clone IT2.2) (PharMingen) was coated (1 ng/100 μl/well) in 96-well plates (Life Technologies) in 0.1 M phosphate buffer (pH 4.0) (16 hr at 4°C) before incubation for 2 hr at room temperature with PBS/ BSA 1%. After washing, plates were incubated for 16 hr at 4°C with culture supernatant (200 μl/well). Bound CD86 was detected with a biotin-labeled anti-CD86 mAb (clone FUN-1) (PharMingen) followed by incubation with streptavidin-biotinylated HRP (used at 1/5000; Amersham Pharmacia Biotech) and revealed by the substrate o-phenylene diamine (Sigma). CD86 expression in transfected COS cells was also evaluated by Western blotting. Cells were washed in ice-cold PBS before lysis in 10 mM phosphate buffer (pH 7.4) containing 1% Nonidet P40 (Sigma) and protease inhibitors (Boehringer Mannheim). Proteins from 5×10^6 cells were electrophoretically separated on a 10% polyacrylamide gel in reducing conditions and then transferred onto a nitrocellulose membrane (Biorad, Ivry sur Seine, France). After saturation, membranes were incubated with an anti-CD86 mAb (R&D Systems). After washing, membranes were incubated with peroxydase-labeled anti-mouse IgG Ab (Amersham Pharmacia Biotech), and bound antibodies were detected using the ECL system (Amersham Pharmacia Biotech).

Production and Purification of Recombinant CD86ΔTM

The recombinant CD86ΔTM molecule was produced in Sf9 cells. The CD86ΔTM cDNA was inserted in the pAcSecG2T baculovirus transfer vector (PharMingen) into which was introduced an enzymatic cleavage site for the protease 3C (Walker et al., 1994) between the CD86ΔTM and GST fragments. Viral particles were prepared as recommended by the manufacturer. Sf9 insect cells were grown at 27°C in TNM-FH medium (Life Technologies). Recombinant baculovirus was generated in vivo in Sf9 cells using Baculogold viral DNA (PharMingen). Sf9 cells were infected at a multiplicity of infection of 0.5. After 12 hr, the culture medium was changed to serum-free Sf-900-II medium (Life Technologies). The cell culture supernatants

were harvested at 72 hr and analyzed by ELISA for the presence of recombinant CD86ΔTM. Cells and cellular debris were sedimented by centrifugation. The supernatants were 20-fold concentrated and the CD86ΔTM molecule was purified on a GSH-Sepharose column (Amersham Pharmacia Biotech). After an overnight incubation at 4°C under agitation, the column was washed and resuspended with PreScission cleavage buffer before addition of GST-PreScission (Amersham Pharmacia Biotech). After a 4 hr incubation at 4°C under agitation, the unbound fraction was collected and dialyzed against PBS. Protein purity was assessed by Coomassie blue staining and Western blotting.

Binding Experiments

The binding of recombinant CD86ΔTM to CD28 was measured by FACS. COS cells were transfected with pCDNA3.1 containing CD28 or CTLA-4 cDNA. Expression of membrane CD28 and CTLA-4 on transfected cells was verified by FACS analysis using FITC-labeled anti-CD28 and anti-CTLA-4 mAbs (PharMingen). Transfected cells were incubated with 10 μg/ml c-myc-tagged recombinant CD86ΔTM. Bound CD86ΔTM was detected with a biotin-labeled anti-c-myc mAb revealed by FITC-labeled streptavidin (PharMingen). In some experiments, a neutralizing anti-CD86 mAb (PharMingen) or CTLA-4-Ig (Ancell) were added. Results are expressed as mean of fluorescence intensity (MFI) values.

T Cell Activation Assays

T cell activation induced by recombinant CD86ΔTM was measured by [3H]thymidine incorporation and IFNγ and IL-2 production. Human PBMC (2.5×10^5 cells/ml), cultured in 96-well culture plates (Life Technologies) (200 μl/well), were either nonstimulated or stimulated with 100 pg/ml anti-CD3 mAb, 30 pg/ml anti-CD3 plus 10 ng/ml anti-CD28 mAbs, or 100 pg/ml anti-CD3 mAb plus 1–1000 ng/ml recombinant CD86ΔTM. In some experiments, 10 μg/ml of the neutralizing anti-CD86 mAb IT2.2 or the isotype control mAbs (both from PharMingen) were added. After 5 days, cells were pulsed with 0.25 μCi/well [3H]thymidine (Amersham Pharmacia Biotech) for 6 hr. Radioactive incorporation was measured by standard liquid-scintillation counting. Results are given in counts per minute (cpm), in stimulation index (SI) calculated as follows: A/O, where A and O are the cpm values obtained when cells were stimulated or not, respectively, or as a percent of decrease, (A-O/A) × 100, where A and O are the values obtained in the absence or presence of a neutralizing anti-CD86 mAb, respectively. In other experiments, the frequency of naive versus memory proliferating T cells was measured using an FITC-labeled anti-CD45RA or anti-CD45RO mAbs (Dako, Glostrup, Denmark) after labeling with Hoechst 33342 (Molecular Probes, Eugene, OR). To measure lymphokine production, PBMC were cultured in 24-well plates (Life Technologies) at 2×10^6 cells/ml, 2 ml/well and stimulated with 100 pg/ml anti-CD3 mAb, 30 pg/ml anti-CD3 plus 10 ng/ml anti-CD28 mAbs, or 30 pg/ml anti-CD3 mAb plus 100 ng/ml recombinant CD86ΔTM. IFNγ production was measured 24 hr later using Quantiflow Immunoassay kits (BioE, St Paul, MN). Briefly, 100 μl of supernatants were incubated with anti-IFNγ or anti-IL-2 antibody–coated beads. After 2 hr, beads were washed and incubated with PE-conjugated anti-IFNγ or anti-IL-2 antibodies. After washing, beads were analyzed by flow cytometry (FACScan). The fluorescent signal given by the beads was detected in log scale, and the settings of the PE-channel photomultiplier (PMT) were adjusted using the blank beads provided in the kit. The cytokine concentration of the samples was determined by comparing the mean channel fluorescence of PE obtained with the standard curve built by plotting mean channel fluorescence versus cytokine concentration, for IFNγ and IL-2 standards recorded. The IFNγ and IL-2 concentration was given in nanograms per milliliter.

Analysis of Vav Phosphorylation

Jurkat cells were incubated for 5 min with 5 μg/ml c-myc-tagged CD86ΔTM, anti-CD28, or control mAb. Cross-linking was performed using an anti-c-myc or goat anti-mouse Ig antibody. Cell lysates were incubated with 2 μg anti-Vav polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) adsorbed on protein A-Sepharose beads. After incubation, beads were washed five times before resuspension in loading buffer. Aliquots were subjected to SDS-PAGE

and electrotransferred on nitrocellulose membranes. For immunoblotting, membranes were probed with a Vav-specific antibody or an anti-phosphotyrosine mAb (clone 4G10; UBI, Lake Placid, NY). After washing, membranes were incubated with peroxidase-labeled anti-mouse or -rabbit antibodies, and bound antibodies were detected using ECL (Amersham Pharmacia Biotech).

Activation of Influenza-Specific CD8^{+} T Cells

The frequency of activated influenza M protein-specific T cells was evaluated as previously reported (Lalvani et al., 1997). Briefly, PBMC at 1×10^6 cells/ml in 200 μl complete medium were cultured for 6 hr and stimulated with 10 μg/ml HLA-A2-restricted influenza peptide FluM1$_{58–66}$ (GILGFVFTL), in the presence of recombinant CD86TM or 3 μg/ml anti-CD28 mAb (Becton Dickinson). Brefeldin A (Sigma), an inhibitor of intracellular transport that prevents secretion of synthesized cytokines, was added in the last 4 hr. Cells were then washed once in cold PBS and then resuspended in 2 ml PBS containing 1 mM EDTA. After 10 min incubation at 37°C, cells were washed and resuspended in 200 μl PBS/1% BSA/0.01% NaN₃. Cells were then incubated for 30 min at 4°C with an allophycocyanin (APC)-labeled anti-CD8 mAb (Caltag, Burlingame, CA) and either phycoerythrin-labeled anti-CD45RA or -CD45R0 mAb (Dako). After washing, cells were fixed in 4% paraformaldehyde in PBS for 10 min at 4°C and permeabilized by a 15 min incubation at 4°C in PBS/1mM HEPES/0.1% saponin in the presence of 5 μg/ml FITC-labeled anti-human IFNγ mAb or FITC-labeled isotype control mAb (both from PharMingen, San Diego, CA). After two washes in PBS/1mM HEPES/0.01% saponin, cells were analyzed on a FACSvantage (Becton Dickinson). For each sample, 30,000 events were acquired after gating on CD8^{+} cells.

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