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B7-H1 (Programmed Death-1 Ligand) on Dendritic Cells Is Involved in the Induction and Maintenance of T Cell Anergy

Nicole Selenko-Gebauer,* Otto Majdic,* Andreas Szekeres,* Gerald Höfler,† Elisabeth Guthann,* Ulf Korthäuer,‡ Gerhard Zlabinger,* Peter Steinberger,* Winfried F. Pickl,* Hannes Stockinger,* Walter Knapp,* and Johannes Stöckl*

In an effort to identify immunoregulatory molecules on dendritic cells (DC), we generated and screened for mAbs capable of modulating the T cell stimulatory function of DC. A particularly interesting mAb was mAb DF272. It recognizes monocyte-derived DC, but not blood monocytes or lymphocytes, and has profound immunomodulatory effects on DC. Treatment of DC with intact IgG or Fab of mAb DF272 enhanced their T cell stimulatory capacity. This effect on DC was accompanied by neither an up-regulation of costimulatory molecules such as B7.1 (CD80), B7.2 (CD86), and MHC class II molecules nor by an induction of cytokine production, including IL-1, TNF-α, IL-10, and IL-12. Moreover, the well-established inhibitory function of IL-10-treated DC could be reverted with mAb DF272. Even T cells, anergized because of stimulation with IL-10-treated DC, could be reactivated and induced to proliferate upon stimulation with mAb DF272-treated DC. Furthermore, mAb DF272-treated DC favored the induction of a type-1 cytokine response in T cells and inhibited IL-10 production. By using a retrovirus-based cDNA expression library generated from DC, we cloned and sequenced the mAb DF272-defined cell surface receptor and could demonstrate that it is identical with B7-H1 (programmed death-1 ligand), a recently identified new member of the B7 family of costimulatory molecules. Our results thus demonstrate that the mAb DF272-defined surface molecule B7-H1 represents a unique receptor structure on DC that might play a role in the induction and maintenance of T cell anergy. The Journal of Immunology, 2003, 170: 3637–3644.

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2 Address correspondence and reprint requests to Dr. Johannes Stöckl, Institute of Immunology, University of Vienna, Borschegasse 8a, A-1090 Vienna, Austria. E-mail address: johannes.stoeckl@univie.ac.at

3 Abbreviations used in this paper: DC, dendritic cell; PI, propidium iodide; GFP, green fluorescence protein; PD-L1, programmed death-1 ligand.

4 Institute of Immunology, University of Vienna Medical School, Vienna, Austria; 5 Department of Pathology, Karl Franzens University, Graz, Austria; and 6 Novartis Research Institute, Vienna, Austria.

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Materials and Methods

Media and reagents

The cell culture medium RPMI 1640 (Life Technologies, Paisley, Scotland) was supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 10% FCS (Sigma-Aldrich, Vienna, Austria). The ecotropic retroviral packaging cell line Phoenix (a kind gift from G. Nolan (Stanford University, Stanford, CA) and colleagues) was maintained in DMEM containing 2 mM glutamine and 10% FCS. Recombinant human GM-CSF, IL-2, and IL-4 were kindly provided by Novartis Research Institute (Vienna, Austria). IFN-γ was a gift from G. R. Adolf (Ernst Boehringer Institut für Arzneimittelforschung, Vienna, Austria). Recombinant human IL-10 was purchased from R&D Systems (Minneapolis, MN). LPS from Escherichia coli (serotype 0127:B8), PHA, ionomycin, and propidium iodide (PI) were obtained from Sigma-Chemie (Deisenhofen, Germany). Annexin V was provided by Caltag Laboratories (Burlingame, CA).

Antibodies

mAbs DF272 (IgG1) were generated in our laboratory by immunizing BALB/c mice with monocyte-derived DC from healthy donors. Fab of DF272 were prepared using papain and the recommended protocol from Avidchrom Fab kit (Unisyn, San Diego, CA). All preparations were further purified by affinity chromatography with protein A and size fractionation with fast protein liquid chromatography superdex gel filtration (Pharmacia Biotech, Uppsala, Sweden). Purity of Fab was checked and confirmed with...
Proliferation of T cells was monitored by measuring methyl calculated as the percentage of annexin V with annexin V-FITC and PE-labeled CD3 mAb UCHT-1. Apoptosis was detected on a microplate scintillation counter (Packard Instrument, H9262). T cells (10^6) were cocultured with DC (10^5) in 24-well plates (Corning-T cells (10^6) were incubated with or without mAb DF272 or with control mAb M80 in 24-well plates (Corning-Costar, Badhoevedorp, The Netherlands) at 37°C for 24 h. Afterward, apoptosis was assessed by staining with FITC-labeled annexin V and PI and performing flow cytometric analysis. Annexin V-positive and PI-negative cells were scored as apoptotic cells.

T cell proliferation assay

For the primary MLR, allogeneic, purified T cells (1 x 10^5/well) were incubated in 96-well cell culture plates (Corning-Costar) with graded numbers of DC for 4 days. The assay was performed in triplicate, and mAbs were added to the respective cultures at a final concentration of 10 μg/ml. Proliferation of T cells was monitored by measuring [methyl-3H]Tdr (ICN Pharmaceuticals, Irvine, CA) incorporation, added on day 5 of culture. Positive selection was performed by MACS (Miltenyi Biotec) using a RS separation column according to the manufacturer’s recommendations (Qiagen, Hilden, Germany). They were rinsed with Tris buffer and incubated with mAb DF272 or isotype-matched control mAb VIAP diluted in DAKO S 2022

Isolation of mAb DF272-binding cells from the retrovirus-based cDNA library-transduced cell pool and expression cloning

The retrovirus-infected cell pool (5 x 10^5) was washed with PBS containing 1% BSA and incubated with mAb DF272 (10 μg/ml) for 30 min on ice. Positive selection was performed by MACS (Miltenyi Biotec) using a RS separation column according to the manufacturer’s instructions. The recovered fraction was expanded for further sorting, and single cell cultures were obtained by limiting dilution after >70% of the isolated cells stained positive with mAb DF272. cDNA was recovered from a mAb DF272 reactive single cell clone using Tri-Reagent (Sigma-Aldrich). RT-PCR was performed with Stratascript (Stratagene, La Jolla, CA) and the Advantage-GC polymerase system (Clontech Laboratories). Cells were harvested 18 h later, and incorporated [methyl-3H]Tdr was detected on a microplate scintillation counter (Packard Instrument, Meriden, CT).

Apoptosis in T cells upon coculture with DC was monitored by staining with annexin V-FITC and PE-labeled CD3 mAb UCHT-1. Apoptosis was calculated as the percentage of annexin V+ cells gated in the CD3+ fraction.

Restimulation of T cells

T cells (1 x 10^5) were cocultured with allogeneic IL-10-treated or untreated DC (1 x 10^5). After 5 days, T cells were harvested and restimulated with graded numbers of untreated DC from the same donor, as used in the primary MLR. MAbs were added to the respective cultures at a final concentration of 10 μg/ml. Proliferation of T cells was measured 3 days later.

Determination of cytokine production

T cells (10^6) were cocultured with DC (10^5) in 24-well plates (Corning-Costar), adding mAbs at a final concentration of 10 μg/ml. After 4 days, the supernatants of primary stimulation cultures were harvested. The cytokines IL-1β, IL-2, IL-4, IL-10, IL-12p40, IL-12p70, and IFN-γ were measured by sandwich ELISAs using matched-pair Abs (R&D Systems). Abs to human TNF-α were from BD PharMingen (San Diego, CA). The lower limit of detection for IL-2 and IL-12p40 was 50 pg/ml; for IL-1β, IL-4, IL-10, IL-12p70, TNF-α, and IFN-γ, it was 20 pg/ml. Standards consisted of human recombinant material (R&D Systems). Assays were performed in duplicates according to the recommendations of the manufacturer. Statistical analysis of the measured values was performed by paired Student’s t test.

Retroviral cDNA expression library

A cDNA library from monocyte-derived DCs that had been constructed in the retroviral vector CRUS-ires-GFP (a kind gift from Rigel Pharmaceuticals, South San Francisco, CA) featuring CMV promoter-driven expression of the cDNA along with green fluorescence protein (GFP) expression from an internal ribosomal entry site, packaged by Phoenix cells into ecotropic virus particles that were then used to infect target cells. For transfection of the library, ecotropic packaging cells (Phoenix-E) at 50% confluency were harvested by trypsinization, washed, and 6 x 10^6 were added to a mixture of 100 μl DMEM containing 1% NuSerum culture supplement (Genome Therapeutics, Waltham, MA), 200 μg/ml DEAE-dextran, 25 μg chloroquine diphosphate, and 60 μg of the CRUS retroviral vector DNA representing the cDNA library. After incubation for 2 h at 37°C, cells were spun down and resuspended in 20 ml DMEM containing 10% FCS and seeded in culture dishes. After 72 h of culture at 32°C, the supernatant was harvested, supplemented with 10 μg/ml hexadimethrine bromide (Sigma-Aldrich), and added to BWS147 target cells (1 x 10^6/ml) in RPMI 1640 medium containing 10% FCS. Cells were incubated for 24 h (37°C) and then used for the selection procedure.

Immunohistochemistry staining and tissue preparation

A microtissue array was used to ensure uniform conditions for immunohistochemical staining (17). Representative areas from normal lymph node, spleen, tonsil, thymus, skeletal muscle, cardiac muscle, placenta, and lung specimens were marked on H&E stained slides. The corresponding areas were microdissected and embedded in a paraffin block.

Immunohistochemistry

Following deparaffinization and rehydration, slides were boiled in a microwave oven in DAKO S 1699 target retrieval solution (DAKO, Glostorp, Denmark). They were rinsed with Tris buffer and incubated with mAb DF272 or isotype-matched control mAb VIAP diluted in DAKO S 2022 Ab diluent for 25 min. Slides were incubated with a secondary Ab (DAKO Chemate Peroxidase/DAB Rabbit/Mouse Kit K5001) for 10 min. Endogenous peroxidase was blocked using 3% H2O2 in methanol. After incubation with the tertiary Ab (DAKO Chemate Peroxidase/DAB Rabbit/Mouse Kit K5001), color development was controlled microscopically, and slides were counterstained using Mayer’s hemalum.
**Results**

**mAbs DF272 recognize a functional receptor on DC**

The aim of this study was to identify cell surface structures on DC with immunoregulatory capacity. For this purpose we immunized mice with human DC to obtain mAbs, which showed reactivity with DC and were capable of modulating the function of DC. mAb DF272 fulfilled these criteria.

Results presented in Fig. 1 demonstrate that mAb DF272 recognizes an Ag expressed on DC but not on resting peripheral blood monocytes or lymphocytes. Expression of the DF272-defined cell surface molecule on DC was further up-regulated upon stimulation of DC. Surprisingly, this effect was not only seen with the maturation-inducing agent LPS (Fig. 1a), but the effect was also observed with the immunosuppressive cytokine IL-10 (Fig. 1b), which usually prevents the induction or up-regulation of typical DC activation markers such as CD83 (16).

To analyze whether binding of mAb DF272 affects the T cell stimulatory function of DC, we added mAb DF272 to DC and used them to activate allogeneic T cells. As can be seen in Fig. 2, addition of mAb DF272 enhanced the T cell proliferative response to immature DC, whereas mAb DF272 had little impact when mature DC were used as stimulators. To test whether blockade of an apoptotic signal for T cells with mAb DF272 might contribute to this elevated T cell proliferation phenomenon, we monitored programmed cell death in our MLR cultures by annexin V staining. We found in four experiments that the number of annexin V+ T cells in absence or presence of mAb DF272 (6.0 ± 2.6% vs 7.8 ± 3.6%, respectively) was only marginally changed. The mAb effect is seemingly at the DC side, because the same enhancement was detected when DC were pretreated with mAb DF272 and washed before coculture with T cells (data not shown). This enhanced T cell proliferation was also seen when Tetanus toxoid-pulsed and purified protein derivative-pulsed DC were used to activate Ag-specific, autologous T cells (data not shown).

Induction of DC activation/maturation upon mAb DF272 binding does not seem to be responsible for this enhanced accessory function. DC treated with mAb 272 did not show elevated expression of CD80, CD86, or HLA-DR, typical signs for activated DC (Fig. 3A). In addition, mAb DF272 neither induced cytokine production (IL-1, TNF-α, IL-12, IL-10) in DC nor affected the viability of DC (data not shown). Most importantly, treatment of DC with Fab of mAb DF272 enhanced their stimulatory capability as well as intact Ab (Fig. 3B). Thus, blocking of the DF272-defined Ag on DC with our mAb significantly enhances T cell stimulation of these cells without detectable signs of DC activation.

**Binding of mAb DF272 to inhibitory IL-10-treated DC restores their stimulatory capacity**

The finding that binding of mAb DF272 to DC enhances their stimulatory capacity suggested that DF272 blocks the function of a potentially inhibitory cell surface structure expressed on DC. This idea was further supported by the observation that the immunosuppressive cytokine IL-10 induced expression of the DF272-defined receptor. Treatment with IL-10 has been shown to reduce the stimulatory capacity of DC (5, 16, 18). Thus, we wondered whether the DF272-defined, putative inhibitory receptor might contribute to the anergizing quality of IL-10-treated DC. To test this possibility, we stimulated allogeneic T cells with IL-10-treated DC in the presence of mAb DF272. We found that IL-10-treated DC are poor stimulators of primary, allogeneic T cells (Fig. 4). Under our test conditions, 10 times more IL-10-treated DC were required to achieve a similar level of T cell proliferation compared...
with untreated DC. However, upon addition of mAb DF272, the poor stimulatory capacity of IL-10-treated DC was reverted, leading to results similar to untreated DC (Fig. 4). Thus, the DF272-defined structure on DC appears to transmit inhibitory signals to T cells, which can be blocked with mAb DF272.

The DF272-defined molecule is involved in the maintenance of anergy

It is well established that IL-10-treated DC are not only poor stimulators of T cells but are capable of inducing Ag-specific anergy in T cells (5, 16, 18). This anergic state cannot be overcome when T cells are restimulated with untreated DC. To test whether blocking of the DF272-defined molecule on DC might enable these cells to break this hypoproliferative state, allogeneic T cells were stimulated first with either IL-10-treated DC or untreated DC and subsequently restimulated with untreated DC from the same donor as used in the primary response. We observed that T cells stimulated in the primary response with IL-10-treated DC showed a reduced proliferative response (Fig. 5a) compared with restimulation of T cells primed with untreated DC (Fig. 5b). This hypoproliferative response was reversible by adding exogenous IL-2 (data not shown). Importantly, addition of mAb DF272 to DC in the secondary response also resulted in reconstitution of T cell proliferation of the anergized T cells (Fig. 5a). This effect was not observed with the control mAb M80. Taken together, these results indicate that the DF272-defined molecule on DC is involved in the induction and maintenance of anergy in T cells by IL-10-treated DC.

Blocking of the surface receptor molecule recognized by mAb DF272 on DC inhibits IL-10 and favors type-1 cytokine production in T cells

The profound effects of mAb DF272 binding to DC on T cell proliferation raised the question of whether the DF272-defined molecule might also be involved in regulating cytokine production in T cells. To analyze this possibility, we cocultured T cells with allogeneic DC treated with mAb DF272.

We observed that T cells stimulated with mAb DF272-treated DC released significantly more IL-2 and IFN-γ than those stimulated with untreated DC (Fig. 6). In contrast, production of IL-4 and IL-10 was reduced (41 ± 26% and 46 ± 22%, respectively) upon activation with DC pretreated with mAb DF272. Thus, the DF272-defined Ag on DC appears to favor IL-10 and IL-4 production. Blocking this signal with mAb DF272 induces a type-1 cytokine release pattern.

The mAb DF272-defined cell surface molecule is B7-H1 (PD-L1)

To identify the molecule recognized by the mAb DF272, we used a retroviral expression cloning approach (10, 11). A cDNA library from monocyte-derived DCs had been constructed in the retroviral vector pLenti 5 (10). The vector allows expression of recombinant proteins in DCs and provides stable cell lines, which can be analyzed for functional activity. We used a retroviral expression cloning approach to identify the molecule recognized by mAb DF272. The results are shown in Fig. 7. The left panel shows a photograph of the cell lines that were selected for their expression of the recombinant protein.

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vector CRU5-IRES-GFP featuring CMV promoter driven expression of the cDNA along with GFP expression from an internal ribosomal entry site. The vector was packaged by Phoenix cells into ecotropic virus particles that were then used to infect target cells. From the transduced cells, those expressing the respective receptor structure recognized by mAb DF272 were isolated by MACS and used for RNA preparation. RNA was reversibly transcribed, and the retrovirus-encoded cDNA insert was PCR amplified. The PCR product was cloned into the mammalian expression vector His-TOPO. To prove the specificity of the inserted cDNA coding for Ag, the plasmids were transfected into 293T cells and stained for mAb DF272 reactivity (Fig. 7A). The cDNA leading to the expression of the molecule recognized by mAb DF272 was sequenced and revealed a length of 1564 bp with an open reading frame of 972 bp. A BLAST similarity search at the National Center for Biotechnology Information showed 100% identity to B7-H1 (PD-L1) (12). Results presented in Fig. 7B demonstrate that mAb DF272 specifically reacts with B7-H1+ transductants, but not with CD80+ transductants.

Expression of B7-H1 in peripheral tissue

With mAb DF272 we then investigated B7-H1 protein expression in peripheral tissues including human tonsil, lymph node, thymus, spleen, colon, skin, lung, placenta, heart, and skeletal muscle. Results presented in Fig. 8A demonstrate that B7-H1 was strongly

FIGURE 6. Engagement of the mAb DF272-defined molecule on DC modulates cytokine production in T cells. For detection of cytokine production, DC were used to stimulate allogenic, purified T cells at a ratio of 1:10 in the presence of mAb DF272 and mAbs against CD80 (DAL-1) and CD86 (BU63). After 4 days, supernatants were harvested and cytokines were analyzed by ELISA. The figure shows mean values ± SD of four experiments.

FIGURE 7. mAb DF272 recognizes B7-H1. A. The cDNA recovered from a mAb DF272-reactive single cell clone was sequenced and revealed 100% identity to B7-H1 (PD-L1). The cDNA was used to transfet 293T cells to confirm specific reactivity of mAb DF272 (green histograms) with transfected cells (a) compared with untransfected cells (b). Reactivity was analyzed by flow cytometry and fluorescence microscopy. The isotype-matched mAb VIAP (open histogram) was used for negative control staining. B. Staining of mAb DF272 with a cloned B7-H1+ transductant and for control with a CD80+ transductant. The figure shows overlay histograms of the indicated mAbs (filled) and the negative control mAb VIAP (open).
expressed on various human epithelia. For instance, squamous epithelia from human tonsil, cylindrical epithelium from the colon, cells from the basal layers of squamous epithelium in the skin, and Hassall’s bodies in the thymus were recognized by mAb DF272 as well as bronchial epithelial and goblet cells from lung sections (data not shown). No such reactivity was found in stainings with the isotope-matched, control mAb VIAP (Fig. 8Aa). mAb DF272 also reacted with syncytiotrophoblasts from human placenta and some keratinocytes in human skin (data not shown). Interestingly, strong B7-H1 expression was observed in a perinuclear dot-like pattern in heart and skeletal muscle cells, suggesting intracellular localization.

Furthermore, B7-H1 expression was found on macrophages as well as on T cells and B cells in lymphoid organs (tonsil, lymph node, spleen) and mucosal tissues (lung, intestine) (Fig. 8Aa). Freshly isolated peripheral blood-mononuclear cells were not recognized (Figs. 1 and 8, B and C). This seems not to be a discrepancy, however. Upon activation of monocytes or lymphocytes, both cell types could be induced to express B7-H1 as it was found on tissue leukocytes (Fig. 8Aa). Thus, B7-H1+ lymphocytes in tissues seem to resemble activated cells.

**Discussion**

Dendritic cells are a family of professional APC with an unmatched ability to interact with and activate T cells (19–21). There is accumulating evidence that DC not only efficiently stimulate T cell activation but also regulate T cell responses (4, 5, 22). There are several known receptor structures on DC that play a critical role in the stimulatory function. However, little is known about cell surface structures involved in the regulation of T cell responses. In this study we report the identification of such a cell surface receptor on DC and demonstrate for the first time that B7-H1 (PD-L1), a recently described new member of the B7 molecule family (12–14, 23) on DC promotes the induction and also the maintenance of anergy in T cells.

We conclude these functional characteristics of B7-H1 on the basis of our results with intact IgG as well as with univalent Fab of mAb DF272. With both preparations, blocking of B7-H1 on DC
was found to enhance T cell stimulation by immature DC and was capable of reverting the poor stimulatory capacity of IL-10-treated DC. Originally, B7-H1 was identified as a costimulatory receptor structure promoting T cell proliferation (12). This costimulatory effect was detectable with B7-H1-transfected COS cells as well as with immobilized B7-H1 fusion proteins combined with suboptimal doses of anti-TCR Abs. In contrast, Freeman et al. (13) demonstrated an immunoinhibitory function for B7-H1. In their experiments, immobilized B7-H1 fusion proteins inhibited T cell proliferation induced by CD3 mAbs. Moreover, Dong et al. (24) reported an apoptosis-inducing property of B7-H1 on tumor cells for cytotoxic T cells. Thus, similar “professional APC-free” T cell activation protocols led to opposite results that are still a matter of debate (25). However, our findings presented in this study clearly revealed that B7-H1 functions as an inhibitory receptor structure on DC.

We believe that the postulated inhibitory signals from B7-H1 are counterbalanced in their activity by the plethora of additional coreceptors expressed on DC. We have shown for instance that B7-H1 is strongly up-regulated on mature DC. Nevertheless, mature DC are the most potent T cell stimulators known (6, 26), and blocking of B7-H1 with mAb DF272 had little effect on them. Because mature DC express high levels of MHC molecules or signal 1 and also abundantly express costimulatory molecules such as B7.1 (CD80), B7.2 (CD86), or signal 2, it seems that B7-H1-derived inhibitory signals are overruled under such conditions and become detectable only when signals 1 and/or 2 are low. This is the case with IL-10-treated DC, which are poor stimulators of T cells (5, 16, 18). The blocking of B7-H1 on these APC reconstitutes the T cell stimulatory quality of DC, indicating that B7-H1 signals are more critical in this case. Thus, we hypothesize that B7-H1 plays a more important role when signals 1 and/or 2 are moderate on APC. This idea is supported by the observation that CD28 cosignaling prevents the inhibitory role of B7-H1 fusion proteins at suboptimal doses of CD3 mAbs or by the finding that inhibitory B7-H1 effects were not found in the presence of large amounts of CD3 mAbs (13). Thus, negative signals from B7-H1 seem to set a threshold for T cell activation on a fully immunocompetent DC.

Yet, B7-H1 may not only become critical in the case of immunocompromised DC but seems to play an important role in controlling anergized T cells through DC. We observed that T cells that were anergized by stimulation with IL-10-treated DC started to proliferate again when they were restimulated with DC treated with mAb DF272. This finding suggests that B7-H1-derived signals might promote the maintenance of an established anergic state in T cells. This could be explained by an increased expression of PD-1 or additional, so far undefined, ligands of B7-H1 on activated T cells (13, 24, 27, 28), rendering previously activated T cells more susceptible to B7-H1 effects than resting T cells (29).

The underlying mechanisms for the profound effects on T cell proliferation induced by blocking B7-H1 on DC are not yet understood. Modulation of the cytokine production pattern in T cells through B7-H1 might certainly be involved. For instance, we observed that the blocking of B7-H1 on DC with mAb DF272 reduced the amount of IL-10 and IL-4 released by cocultured T cells. In contrast, IFN-γ and IL-2 production was strongly increased under these conditions. These results suggest that B7-H1 cosignaling of DC might not only increase IL-10 production (12, 14), but reveal a more T cell-directive function. B7-H1 seems to promote type-2 T cell responses and alternatively regulates the expression of critical factors for T cell proliferation, IL-2 and IL-10. The finding that B7-H1 signaling might enhance IL-10 production, but inhibit IL-2 production, could explain how B7-H1 controls T cell anergy. However, it remains to be determined whether this effect is directly transferred from the DC to the Ag-specific T cell or indirectly, i.e., via activation of regulatory T cells through B7-H1.

Interestingly, the observed immunoregulatory potency of B7-H1 may not only be mediated via DC but may also be exploited by nonimmune cells. Expression of B7-H1 in extracts of nonhemopoietic tissues has been detected at the RNA level (12–14, 25, 29). We could confirm these findings and demonstrate that several tissues express B7-H1. Of particular interest in this respect is that epithelial cells as well as muscle cells were found to strongly express B7-H1 protein. Because these nonimmune cells are not usually equipped with costimulatory molecules like CD80 or CD86 (25), it is thus tempting to speculate that B7-H1 on such cells may have profound inhibitory effects on adjacent T cells. This could also play a role in the induction and maintenance of anergy in peripheral T cells.

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