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Minimal costimulatory requirements for T cell priming and TH1 differentiation: Activation of naive human T lymphocytes by tumor cells armed with bifunctional antibody constructs

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Abstract

Direct priming of naive human CD8+ and CD4+ T cells by tumor cells devoid of any intrinsic antigen presentation properties, but passively armed with recombinant proteins mediating primary and costimulatory T cell signals, was investigated. Bifunctional antibody constructs were used to specifically target costimulatory molecules such as B7-1, B7-2 and LFA-3 to the epithelial cell adhesion molecule (EpCAM), a surface antigen successfully used as target for antibody therapy of minimal residual colorectal cancer. T cell priming was monitored by flow cytometric analysis of CD45 isoform expression and confirmed by measuring typical effector functions of primed T cells known to be absent from naive T lymphocytes. Accordingly, CD8+ T cells were tested for cytotoxic activity and secretion of TNF-alpha, while secretion of IFN-gamma, IL-5 and IL-4 was determined for CD4+ T cells. B7, known to be required for the initial activation of naive T cells, also proved to be sufficient for T cell priming when present as the only costimulatory molecule together with an appropriate primary signal. The requirement of dendritic and other antigen presenting cells (APCs) for T cell priming through non-APCs such as tumor cells could be ruled out. Under minimal priming conditions, naive CD4+ T cells were found to exclusively enter the TH1 developmental pathway, while several factors thought to favor TH2 polarization, like weak primary signals and B7-2 versus B7-1 costimulation, could be excluded as dominant TH2 promoters.

Introduction

The process of antigen presentation as executed by dendritic cells includes several temporally discrete steps, such as the uptake of antigen, its processing and the priming of naive T lymphocytes. Tumor cells are notoriously

deficient in these activities and thus do not frequently elicit an immune response that leads to tumor rejection. The loss of MHC molecules holds a prominent position (1) among the various explanations given for the failing immunogenicity of tumors. Moreover, antigen presentation by tumor cells may render naive T cells anergic or apoptotic when costimulatory signals that are typically provided by professional antigen presenting cells (APC) are lacking. Prototypes of such costimulatory ligands are members of the B7 family that bind to the CD28 counter-receptor on T cells (2). Accordingly, tumor cell lines deficient in APC properties could be demonstrated to regain immunogenicity after transfection with the B7-1 gene (3). The resulting T cell response was also directed against untransfected tumor cells, suggesting that the parental tumor by itself failed to induce T cell priming despite antigen presentation. Further experiments in mouse models revealed two different pathways of T-cell priming: (i) Direct interaction of T-cells with antigens presented on B7-1 transfected tumor cells and (ii) cross presentation of target antigen through professional APCs. However, minimal costimulatory requirements for direct priming of naive T-cells could not be defined from these experiments since interference by other cells or molecules and/or indirect antigen presentation could not be excluded (4). Therefore, we investigated whether tumor cells by themselves can be induced to prime naive human T cells and whether B7 as such suffices to set off the priming process without other costimulatory requirements. Although in several *in vitro* studies the attempt was made to display B7 molecules on the surface of tumor cells, either by transfection, passive insertion into the cell membrane (5) or specific targeting to other cell surface molecules (6,7,8), the effect of this manipulation was studied only with regard to unspecific parameters such as the proliferation of unseparated T cells. However, the priming of purified naive T cells by the transferred B7 was not addressed.

The present work was designed to substitute lacking costimulatory signals on tumor cells by passively arming them with recombinant protein constructs that convey full costimulatory activity. B7-1 was specifically anchored on the cell membrane by a novel antibody-based targeting molecule, designated heterominibody, comprising the two N-terminal B7 domains and an anti-tumor scFv antibody fragment. The construction of the heterominibody is described and its costimulatory activity is compared to that of targeted B7-1, B7-2 and LFA-3 single-chain constructs. In addition a CD3-directed bispecific single-chain antibody was used that substituted for the primary signal mediated by the clonotypic T cell receptor (TCR). In a series of *in vitro* experiments, we show that the combination of the TCR- and B7-derived signals triggered by the two recombinant proteins provides a full-fledged complement of activation signals that sets off the priming process in naive human T lymphocytes and at the same time triggers the differentiation pathway towards the TH1 subset. Additional factors thought to influence TH1/TH2-differentiation were tested under the defined minimal priming conditions. Possible applications of targeted costimulatory molecules in cancer immunotherapy are discussed in view of the positive priming experiments.

Results

Novel heterodimeric antibody constructs for the targeting of costimulatory molecules

A recombinant antibody construct designated heterominibody was developed that allows specific targeting of human B7-1 to the human EpCAM antigen on epithelial tumor cells. As shown in Figure 1A, the construct consists of two different polypeptide chains that heterodimerize during expression and secretion by mammalian host cells through the constant immunoglobulin domains Ckappa and CH1gamma1. Human Ckappa was fused via the flexible upper hinge region of human IgG3 to the C-terminus of an scFv fragment derived from the EpCAM-specific monoclonal antibody M79. In the same way, human CH1gamma1 was joined to the C-terminus of the extracellular part of human B7-1. For purification and detection purposes, a C-terminal polyhistidine tag was added to the CH1 domain. Interaction of the immunoglobulin heavy chain binding protein (BiP) with CH1 was envisaged to prevent secretion of CH1 polypeptide chains that have not paired with their Ckappa

counterpart (9). Accordingly, histidine tag-based purification of recombinant protein from culture supernatant proved to be specific for the desired heterodimer, although unpaired Ckappa polypeptide chains were detectable in the supernatant (data not shown). Stable transfection of CHO cells with both polypeptide chains followed by gene amplification yielded 0.5 mg purified heterominibody per liter culture supernatant. On the SDS-PAGE (Figure 2A), the recombinant protein exhibited a molecular weight of 105 kDa under non-reducing conditions; under reducing conditions, two bands of 45 kDa and approx. 60 kDa appeared, corresponding to the Ckappa and the CH1 polypeptide chains respectively. Thus the heterodimeric structure of the molecule, as well as the formation of a covalent disulfide bond between Ckappa and CH1, could be confirmed. Moreover the presence of a broad 60 kDa band, as compared to the distinct 45 kDa band, indicates glycosylation of B7-1.

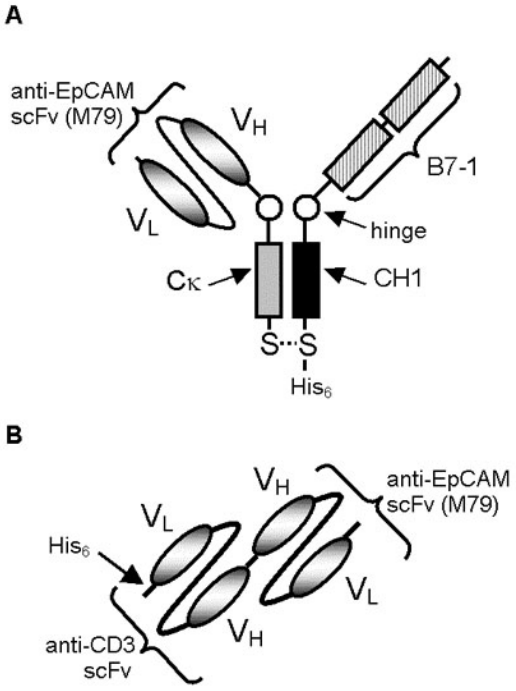


Figure 1. Structure of constructs. (A) The EpCAM-specific heterodimeric B7-1 construct consists of: B7-1, the extracellular part of human B7-1 comprising two Ig-like domains. Hinge (-o-), the upper hinge region of human IgG3. Ckappa, constant region domain of human Ig kappa light chain. CH1, the first constant region domain of the human IgG1 heavy chain comprising at its C-terminus (i) the cysteine residue that forms the interchain disulfide bond (-S-S-) with the C-terminal cysteine of Ckappa, followed by (ii) six histidine residues (His_6). Anti-EpCAM scFv (M79), scFv fragment of EpCAM-specific monoclonal antibody M79, comprising the variable regions of the corresponding Ig light and heavy chains, denoted VL and VH respectively, connected by a 15-amino acid glycine-serine linker $(G_4S)_3$. (B) Bispecific single-chain antibody (bscAb) EpCAM x CD3 (without a Flag tag) providing T cells with the TCR signal through simultaneous binding of its anti-CD3 scFv arm (to the CD3 component of their TCR complex) and of its anti-EpCAM scFv arm derived from monoclonal antibody M79 (to EpCAM-positive cells). VL and VH, the variable regions of the anti-EpCAM or anti-CD3 Ig light and heavy chains respectively. His_6, C-terminal tag consisting of 6 histidine residues.

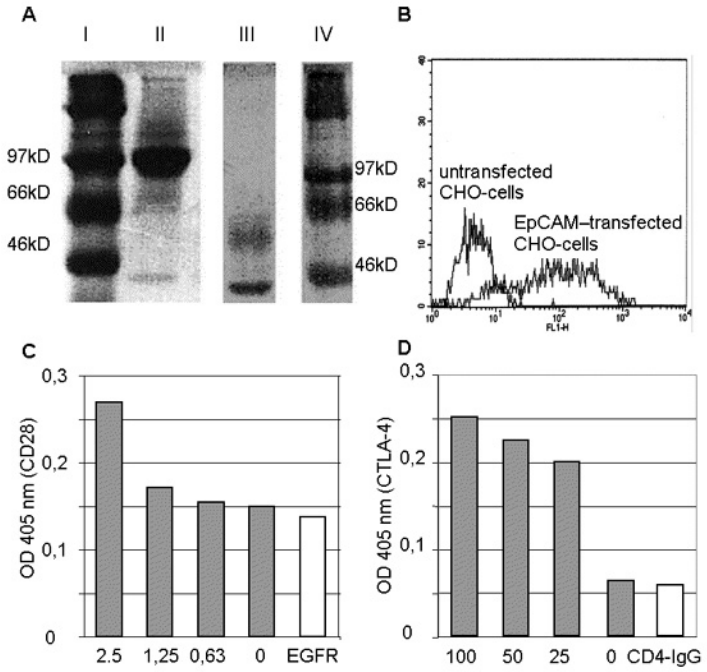


Figure 2. Properties of the EpCAM-specific heterodimeric B7-1 construct. (A) SDS-PAGE under non-reducing (lanes I and II) and reducing conditions (lanes III and IV). Lanes I and IV, molecular weight markers. Lanes II and III, purified heterodimeric B7-1 construct. (B) Flow cytometric analysis of the heterodimeric B7-1 construct on untransfected CHO cells (negative control) and CHO cells transfected with a full length cDNA encoding the human EpCAM antigen. (C) ELISA on immobilized recombinant CD28. The OD_{405} values shown correspond to different concentrations of purified heterodimeric B7-1 construct (in µg/ml). Immobilized recombinant EGF receptor (EGFR) incubated with 2.5 µg/ml heterodimeric B7-1 construct served as a negative control. (D) ELISA on immobilized CTLA-4-IgG fusion protein. The OD_{405} values shown correspond to different concentrations of purified heterodimeric B7-1 construct (in µg/ml). Immobilized CD4-IgG fusion protein incubated with 100 µg/ml heterodimeric B7-1 construct served as a negative control.

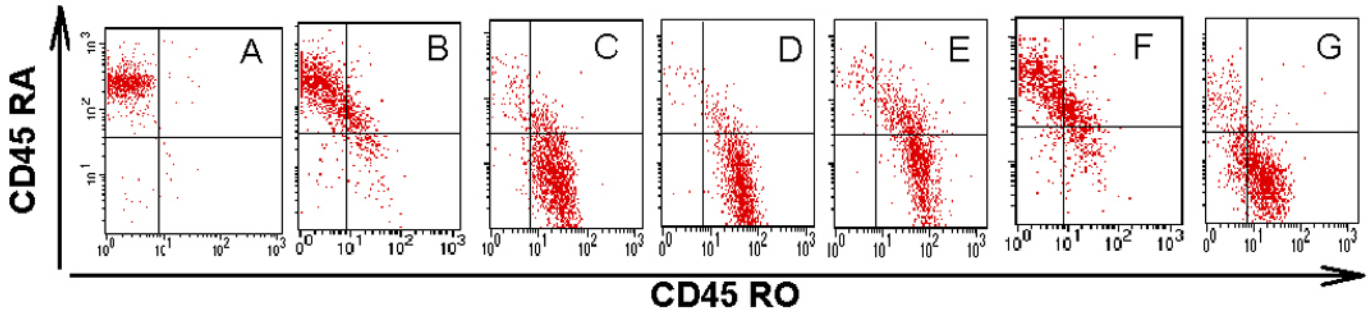
The recombinant B7-1 heterominibody exhibited specific binding to the human EpCAM antigen as demonstrated by flow cytometry on EpCAM-transfected Chinese hamster ovary (CHO) cells (10); in contrast, no binding was detectable on untransfected control cells (Figure 2B). Binding of B7-1 heterominibody to the B7 ligand CD28 was analyzed by ELISA on immobilized recombinant CD28; as negative control, immobilized recombinant human epidermal growth factor receptor (EGFR) was used. As shown in Figure 2C, increasing concentrations of B7-1 heterominibody gave signals above background on CD28 but not on the irrelevant protein, confirming the specific interaction of the B7 construct with CD28. Specific binding of the B7-1 heterominibody to the second B7 ligand CTLA-4 could be confirmed by ELISA on immobilized CTLA-4-IgG fusion protein with immobilized CD4-IgG fusion protein serving as negative control (Figure 2D).

In vitro priming and TH1/TH2-differentiation of naive CD4+ T cells

Naive CD4+ T lymphocytes with the typical phenotype CD45RA+RO- were purified from the peripheral blood of healthy donors and incubated with irradiated EpCAM-transfected CHO cells as stimulator cells (11,12,13). The primary signal was mediated by the bispecific single-chain antibody (bscAb) EpCAM x CD3 (Figure 1B), imitating specific antigen recognition through the TCR; the second or costimulatory signal was mediated by the EpCAM-specific B7-1 heterominibody. Non-human stimulator cells were used in order to avoid background signals that

may arise with human stimulator cells incidentally expressing costimulatory receptors. T cell priming was monitored by flow cytometry on days 4 and 6 by simultaneously measuring the expression of CD45RA and CD45RO. In the presence of both B7-1 heterominibody and bscAb EpCAM x CD3 at the highest concentration (250 ng/ml), the CD45 phenotype of almost the entire population of naive T cells changed to that of primed T cells, i.e. CD45RA-RO+, within 6 days (Figure 3C). By day 4, a smaller percentage of T cells had already completed the phenotype switch, as was observed by day 6 with lower concentrations of bscAb EpCAM x CD3 (data not shown). Although naive T cells seemed to initiate modulation of CD45RA/RO expression with 250 ng/ml bscAb EpCAM x CD3 alone, they obviously failed to complete the phenotype switch (Figure 3B). Importantly, higher concentrations of bscAb EpCAM x CD3 (up to 4 µg/ml) still could not induce substantial changes in CD45 isoform expression in the absence of costimulation.

1. signal	-	+	+	+	+	+	+
2. signal	-	-	heterodimeric B7-1	single chain B7-1	single chain B7-2	single chain LFA-3	sc B7-1 + sc ICAM-1



IFN γ	-	-	+	+	+	-	+
IL4/IL5	-/-	-/-	-/-	-/-	-/-	-/-	-/-

Figure 3. Flow cytometric analysis of CD45 isoform expression. (A) Purified naive CD4+ T cells (no additional MHC II depletion). These were subsequently stimulated for 6 days with EpCAM-transfected CHO cells armed either with (B) 250 ng/ml bscAb EpCAM x CD3 alone, (C) 250 ng/ml bscAb EpCAM x CD3 and 500 ng/ml heterodimeric B7-1 construct, (D) 250 ng/ml bscAb EpCAM x CD3 and 500 ng/ml B7-1 single-chain construct, (E) 250 ng/ml bscAb EpCAM x CD3 and 500 ng/ml B7-2 single-chain construct, (F) 250 ng/ml bscAb EpCAM x CD3 and 500 ng/ml LFA-3 single-chain construct or with (G) 250 ng/ml bscAb EpCAM x CD3, 125 ng/ml B7-1 single-chain (sc) construct and 500 ng/ml ICAM-1 single-chain (sc) construct. The stimulation pattern of the TCR-like signal (signal 1) and costimulation signal (signal 2) is given in the upper panel. The T cells' corresponding IFN-gamma and IL-4/IL-5 expression profiles are shown in the lower panel.

In order to confirm that the switch in surface phenotype actually indicates T cell priming as defined by effector functions, we investigated the secretion of cytokines known to be exclusively expressed by primed, but not by naive, CD4+ T cells. Since priming may further result in TH1- or TH2-polarization, we chose IFN-gamma, typically secreted by primed CD4+ T cells with TH1 phenotype, as well as IL-4 and IL-5, which are only produced by TH2 cells. As shown in Figure 4, CD4+ T cells that underwent the CD45 phenotype switch due to the presence of the bscAb EpCAM x CD3 and the B7-1 heterominibody showed strong IFN-gamma secretion into the culture supernatant. On the contrary, neither IL-4 nor IL-5 could be detected in the supernatant of primed CD4+ T cells (data not shown). As expected, these three cytokines were not detectable when naive CD4+ T cells

were incubated on EpCAM-transfected CHO cells with bscAb EpCAM x CD3 alone. Thus our results indicate that priming of CD4+ T cells, assisted by costimulation through the B7-1 heterominibody, exclusively results in TH1 polarization.

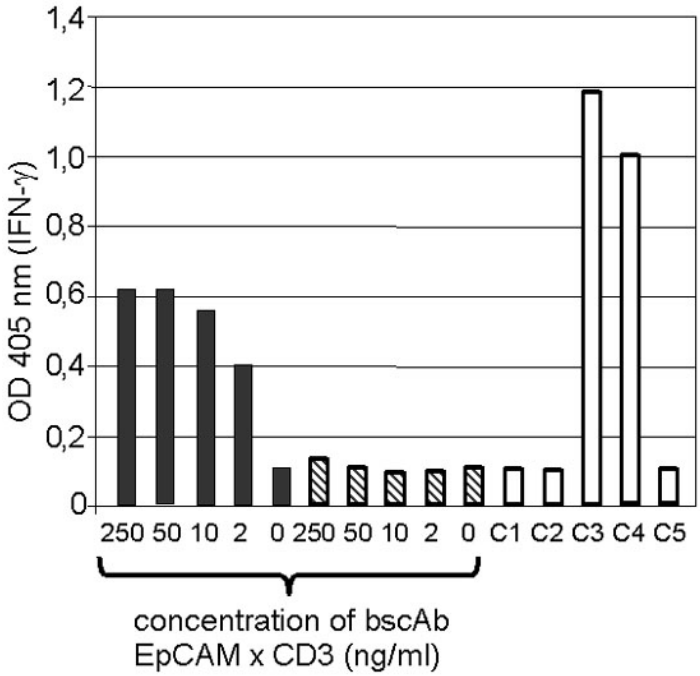


Figure 4. ELISA analysis of IFN-gamma production. IFN-gamma production by purified naive CD4+ T cells (no additional MHC II depletion) after 4 days of stimulation with EpCAM-transfected CHO cells armed either with bscAb EpCAM x CD3 alone, thus providing only a TCR-like primary signal (hatched columns) or with bscAb EpCAM x CD3 and 500 ng/ml heterodimeric B7-1 construct, providing an additional costimulatory or second signal (black columns). Controls (empty columns): C1, purified naive CD4+ T cells incubated with 250 ng/ml bscAb EpCAM x CD3 and 500 ng/ml heterodimeric B7-1 construct in the absence of EpCAM-transfected CHO cells. C2, purified naive CD4+ T cells incubated with 250 ng/ml bscAb EpCAM x CD3 alone in the absence of EpCAM-transfected CHO cells. C3, unseparated PBMC incubated with 250 ng/ml bscAb EpCAM x CD3 and 500 ng/ml heterodimeric B7-1 construct in the presence of EpCAM-transfected CHO cells. C4, unseparated PBMC incubated with 250 ng/ml bscAb EpCAM x CD3 alone in the presence of EpCAM-transfected CHO cells. C5, unseparated PBMC incubated in the presence of EpCAM-transfected CHO cells without bscAb EpCAM x CD3 and heterodimeric B7-1 construct. The OD_{405} values for the IFN-gamma ELISA are shown.

Since targeted B7-1 and B7-2 fusion proteins have been described in the literature (7, 8), we compared the B7-1 heterominibody with EpCAM-specific B7-1 and B7-2 single chain constructs in the CD4+ T cell priming assay. To this end the B7-1 heterominibody was replaced as costimulatory molecule by a B7-1 or B7-2 single-chain construct (Figure 5) at a final concentration of 500 ng/ml. As shown in Figure 3 (C and D), naive CD4+ T cells receiving costimulation through the B7-1 single-chain construct switched to the primed CD45 isoform pattern as efficiently as those costimulated with the B7-1 heterominibody. Furthermore, cytokine analysis of culture supernatants revealed secretion of IFN-gamma in the absence of IL-4 and IL-5 by CD4+ T cells primed through costimulation with the B7-1 single-chain construct. Thus under these conditions targeted B7-1 provided sufficient costimulation for CD4+ T cell priming and induced TH1 differentiation exclusively, irrespective of the molecular format of the B7 construct. Targeted B7-2 also induced the CD45 phenotype switch in naive CD4+ T cells, albeit with slightly lower efficiency (Figure 3E). It is noteworthy that the profile of IFN-gamma, IL-4 and IL-5 secretion was indistinguishable between CD4+ T cells primed through costimulation with targeted B7-1 and those

costimulated with B7-2. Thus, B7-mediated costimulation of CD4+ T cells always led to the exclusive differentiation of TH1 cells irrespective of the nature of the costimulatory signal, i.e. whether it was derived from B7-1 or B7-2. Costimulation of naive CD4+ T lymphocytes by targeted B7-1 or B7-2 was always accompanied by a strong proliferative response as measured in a 5-bromodeoxyuridine (BrdU) incorporation assay (Figure 6). However, the proliferation of naive T cells incubated on EpCAM-transfected CHO cells with bscAb EpCAM x CD3 alone was only slightly above background.

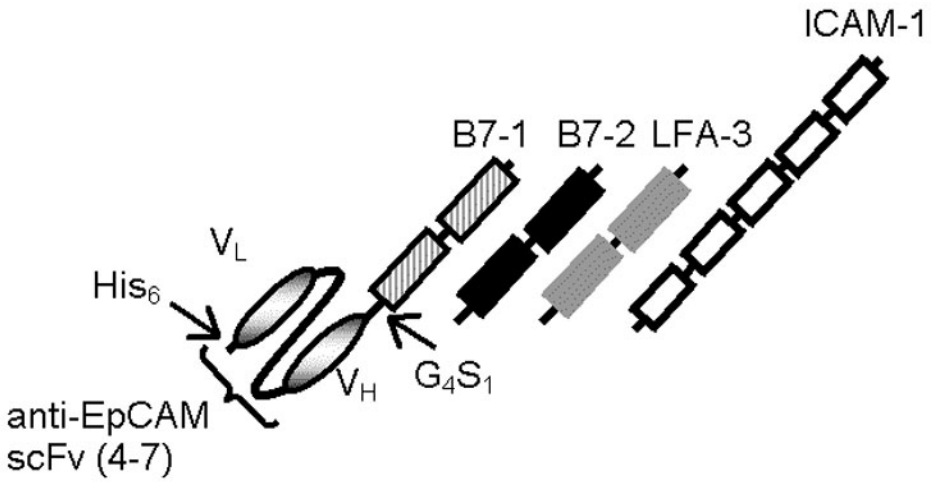


Figure 5. Design of EpCAM-specific costimulatory single-chain constructs. The single-chain constructs consist of: B7-1, B7-2, LFA-3, the extracellular parts of human B7-1, B7-2 and LFA-3 respectively, each comprising two Ig-like domains. ICAM-1, the extracellular part of human ICAM-1 comprising five Ig-like domains. Anti-EpCAM scFv (4-7), the EpCAM-specific scFv antibody fragment 4-7 comprising the Ig heavy and light chain variable regions VH and VL respectively connected by a 15 amino acid glycine-serine linker $(G_4S_1)_3$. His_6, 6 histidine residues at the C-terminus of anti-EpCAM scFv (4-7). G_4S1, 5 amino acid glycine-serine linker connecting B7-1, B7-2, LFA-3 or ICAM-1 with anti-EpCAM scFv (4-7).

In contrast to targeted B7, costimulation of naive CD4+ T cells through LFA-3 mediated by 500 ng/ml of an EpCAM-specific LFA-3 single-chain construct (Figure 5) was not capable of completing the CD45 phenotype switch (Figure 3F). Nevertheless, T cell proliferation was in the range of that induced through costimulation by B7-1 or B7-2 (Figure 6). However, the complete absence of cytokines typically secreted by primed CD4+ T cells, i.e. either IFN-gamma or IL-4 and IL-5, demonstrated that the proliferative response induced by costimulation through LFA-3 was not accompanied by functional T cell priming.

Since ICAM-1 has been speculated to influence TH1/TH2-differentiation, we replaced the B7-1 heterominibody in the CD4+ T cell priming assay by 125 ng/ml B7-1 single-chain construct and 500 ng/ml of an EpCAM-specific ICAM-1 single-chain construct (Figure 5). Under these modified conditions, the naive CD4+ T cells underwent an almost quantitative phenotype switch to the CD45 isoform pattern of primed T cells as expected, but persisted to exclusively follow the TH1 developmental pathway according to the cytokine profile (Figure 3G). The ICAM-1 single-chain construct without targeted B7-1 did not have any effect on CD45 isoform expression, nor did it induce a proliferative response in the CD4+ T cell priming experiment (data not shown).

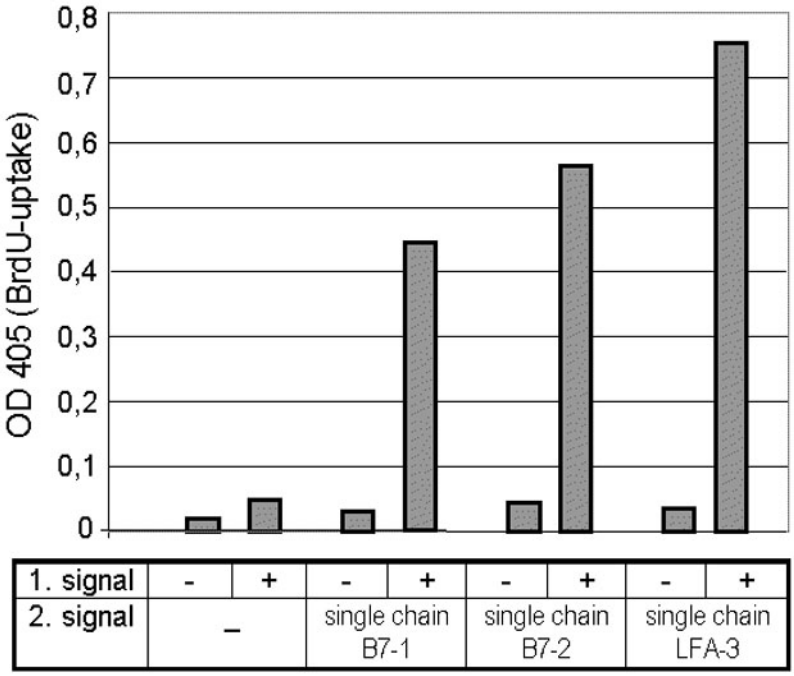


Figure 6. Proliferation analysis. The proliferation of purified naive CD4+ T cells (no additional MHC II depletion) was measured in triplicate by an ELISA-based BrdU incorporation assay after 4 days of stimulation with EpCAM-transfected CHO cells armed with 250 ng/ml bscAb EpCAM x CD3 (+), providing the first TCR-like signal, with or without a second signal mediated by 500 ng/ml EpCAM-specific single-chain B7-1, B7-2 or LFA-3 construct. Negative controls, in which the first and/or second signal are absent, are indicated by (-). The mean OD_{405} values for the uptake of BrdU are shown.

The role of professional antigen presenting cells in direct priming of naive T cells by non-APCs

In order to exclude that residual APCs such as dendritic cells that may still contaminate the preparations of purified naive T lymphocytes contribute significantly to T cell priming in our experiments, we depleted MHC II-positive cells simultaneously with CD45RO+ T lymphocytes in the final step of the purification protocol. The absence of APCs from the preparations of naive T cells was controlled by allogeneic mixed lymphocyte reactions (MLRs). To this end, PBMC, bulk CD4+ T cells prior to CD45RO/MHC II depletion and purified naive CD4+ T cells of the same donor were irradiated and served as stimulator cells. Purified CD4+ T cells from another donor were incubated as responder cells with each of these different populations of irradiated stimulator cells. The proliferation of the responder cells was measured by a BrdU incorporation assay (Figure 7). As expected, PBMC induced strong proliferation of the responder cells. With bulk CD4+ T cells prior to CD45RO/MHC II depletion there was still considerable proliferation indicating the presence of residual APCs. However, the uptake of BrdU induced by purified naive CD4+ T cells from which MHC II-positive cells had been depleted was as low as the negative control, thus demonstrating the absence of APCs in functionally relevant numbers. Therefore we repeated the *in vitro* priming experiment with these naive CD4+ T cells confirmed to be essentially free of contaminating dendritic cells or other APCs. The naive CD4+ T cells were thus costimulated with 500 ng/ml of the EpCAM-specific B7-1 single-chain construct; the primary TCR-like signal was mediated by 250 ng/ml bscAb EpCAM x CD3. In the negative control, bscAb EpCAM x CD3 was omitted.

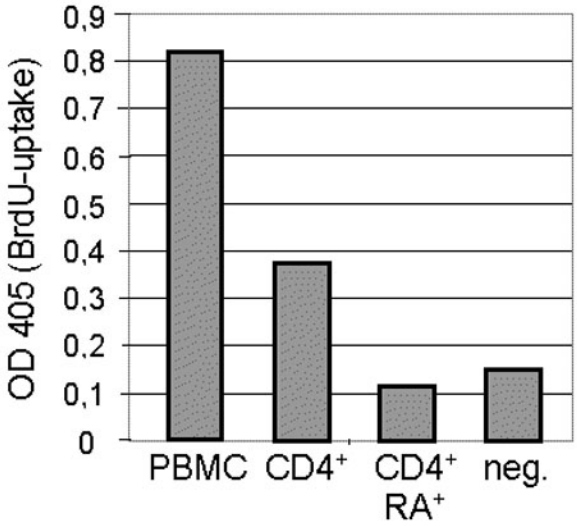


Figure 7. Representative allogeneic MLRs monitoring the depletion of residual APCs for the different steps of the naive CD4+ or CD8+ T cell purification protocol. The proliferation of CD4+ T lymphocytes (responder cells) stimulated for 4 days with irradiated preparations of PBMC, bulk CD4+ T cells (CD4+) or MHC II depleted naive CD4+ T cells (CD4+ RA+) from another healthy donor (stimulator cells) measured in triplicate by an ELISA-based BrdU incorporation assay are shown. As a negative control (neg.), responder cells were incubated without stimulator cells. The mean OD_{405} values for the uptake of BrdU are shown.

As shown in Figure 8, naive CD4+ T cells that exclusively received B7-mediated costimulation and an appropriate primary signal still underwent a quantitative switch to the primed T cell CD45 isoform pattern, despite the confirmed absence of professional antigen presenting cells. The phenotype switch on day 4 represented an intermediate state and was more advanced on day 6. In contrast to naive T cells which were not MHC II depleted, signs of initial CD45RA/RO modulation with 250 ng/ml bscAb EpCAM x CD3 alone could no longer be detected. Thus residual antigen presenting cells may have contributed to some extent to the activation of naive T lymphocytes without actually being required for direct T cell priming through non-APCs. Priming of naive CD4+ T cells, as indicated by the CD45 isoform switch, was confirmed functionally by cytokine analysis. As shown in Figure 9A, CD4+ T cells that underwent the CD45 phenotype switch in the presence of bscAb EpCAM x CD3 and the B7-1 single-chain construct showed IFN-gamma secretion into the culture supernatant. On the contrary, neither IL-4 nor IL-5 could be detected in the supernatant of primed CD4+ T cells in this same experiment. As expected, none of these three cytokines was detectable when naive CD4+ T cells were incubated on EpCAM-transfected CHO cells with bscAb EpCAM x CD3 alone. In order to ensure that TH2 differentiation can actually be induced under these conditions, the *in vitro* priming experiment was also carried out with naive CD4+ T cells in the presence of 100 U/ml IL-4. Indeed IL-4 induced CD4+ T cells to enter the TH2 developmental pathway as indicated by the secretion of IL-5 and the suppressed IFN-gamma production (Figure 9B). Although no external IL-12 was added, intrinsically produced IL-12 may have forced the naive CD4+ T lymphocytes to become TH1-cells. However, no IL-12 was detectable in the culture supernatants on days 4 and 6 using a sensitive ELISA except with PHA-stimulated PBMC (data not shown). Thus the results obtained after depletion of residual APCs confirmed exclusive TH1 differentiation of CD4+ T cells primed through costimulation by targeted B7 in the absence of any accessory signals.

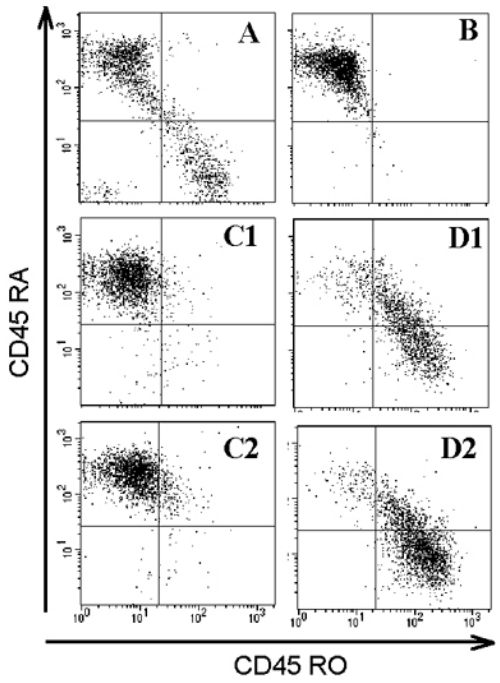


Figure 8. Flow cytometric analysis of CD45 isoform expression. (A) Unseparated CD4+ T cells. (B) APC-depleted purified naive CD4+ T cells. The latter were subsequently stimulated with EpCAM-transfected CHO cells armed either with (C) 250 ng/ml bscAb EpCAM x CD3 alone, thus providing only a TCR-like primary signal, or with (D) 250 ng/ml bscAb EpCAM x CD3 and 500 ng/ml single-chain B7-1 construct providing an additional costimulatory or second signal. C1 and D1 show the results after 4 days and C2 and D2 after 6 days of stimulation respectively.

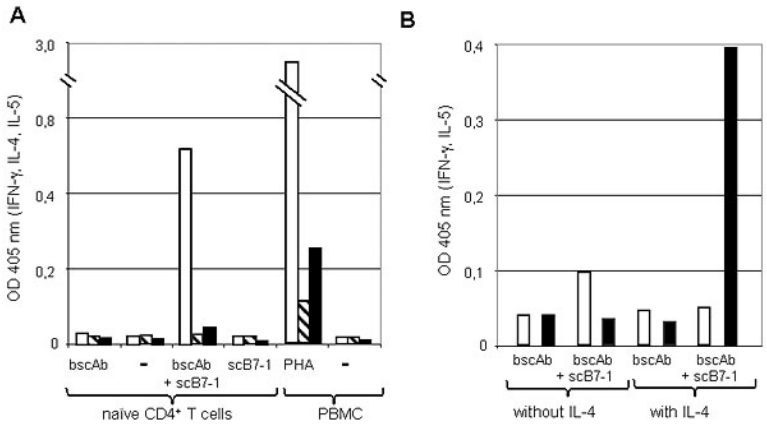


Figure 9. Determination of cytokine production by quantitative ELISA. Open columns, IFN-gamma. Hatched columns, IL-4. Black columns, IL-5. The OD_{405} values for the ELISA are shown. (A) Cytokine production by APC-depleted, purified naive CD4+ T cells after 4 days of stimulation with EpCAM-transfected CHO cells armed with 250 ng/ml bscAb EpCAM x CD3 (bscAb) and/or 500 ng/ml single-chain B7-1 construct (scB7-1). Stimulation by unarmed EpCAM-transfected CHO cells served as negative control (-). Additional controls consisted of PBMC that were stimulated for 4 days with 5 µg/ml PHA or remained unstimulated (-). (B) Cytokine production by APC-depleted, purified naive CD4+ T cells primed in the presence or absence of 100 U/ml IL-4 after 10 days of culture. Abbreviations and concentrations of constructs as in A.

In vitro priming of naive CD8+ T cells

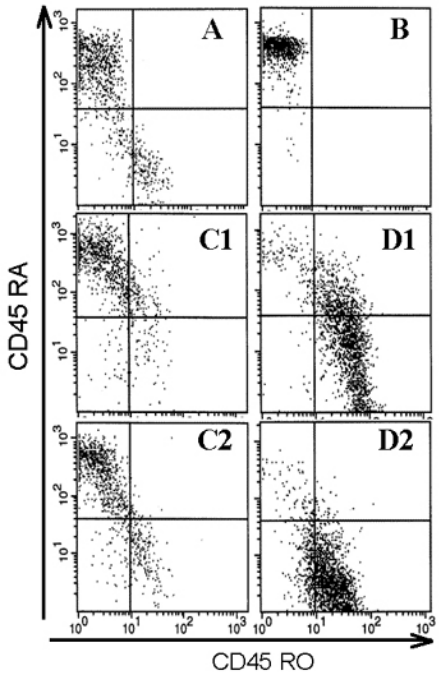


Figure 10. Flow cytometric analysis of CD45 isoform expression. (A) Unseparated CD8+ T cells. (B) Purified naive CD8+ T cells (no additional MHC II depletion). These were subsequently stimulated with EpCAM-transfected CHO cells armed either with (C) 250 ng/ml bscAb EpCAM x CD3 alone, thus providing only a TCR-like primary signal, or with (D) 250 ng/ml bscAb EpCAM x CD3 and 500 ng/ml heterodimeric B7-1 construct additionally providing a costimulatory or second signal. C1 and D1 show the results after 4 days and C2 and D2 after 6 days of stimulation respectively.

Naive CD8+ T cells were obtained from the peripheral blood of healthy donors. Since primed nonproliferating cytotoxic CD8+ T cells share the CD45RA+RO- phenotype with naive CD8+ T lymphocytes, CD11b was used as an additional purification marker in order to eliminate the former T cell subset. Thus, only CD11b- CD8+ T cells entered the purification procedure based on CD45 isoforms, resulting in naive CD8+ T lymphocytes with the CD45RA+RO- phenotype, as was the case for naive CD4+ T cells (14,15). The naive CD8+ T cells were also incubated with irradiated EpCAM-transfected CHO cells acting as stimulator cells. They received the primary TCR-like signal through the bispecific single-chain antibody (bscAb) EpCAM x CD3 and the costimulatory signal from the EpCAM-specific B7-1 heterominibody. T cell priming was monitored by flow cytometry on days 4 and 6 by simultaneously measuring the expression of CD45RA and CD45RO. As observed with naive CD4+ T cells, their CD8+ counterparts also underwent an almost quantitative change in CD45 isoform pattern to that of primed T cells i.e. CD45RA-RO+ in the presence of both the B7-1 heterominibody and 250 ng/ml bscAb EpCAM x CD3 (Figure 10). Lower concentrations of bscAb EpCAM x CD3 reduced the percentage of T cells that completed the phenotype switch (data not shown). The kinetics of the phenotype switch of naive CD8+ and CD4+ T cells proved to be nearly indistinguishable. As expected, EpCAM-transfected CHO cells armed with bscAb EpCAM x CD3 alone failed to prime naive CD8+ T cells according to the CD45 isoform pattern. The BrdU incorporation assay results for the CD8+ T cells resembled those obtained with CD4+ T cells: The switch in CD45 phenotype of CD8+ T lymphocytes was also accompanied by strong T cell proliferation and naive CD8+ T cells incubated on EpCAM-transfected CHO cells with bscAb EpCAM x CD3 alone hardly showed any proliferative response.

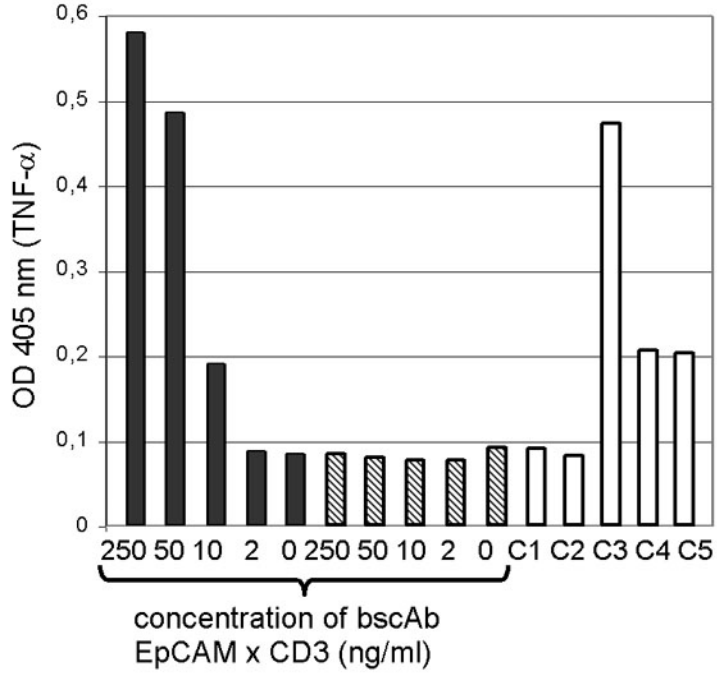


Figure 11. Determination of TNF-alpha production by ELISA. TNF-alpha production by purified naive CD8+ T cells (no additional MHC II depletion) after 4 days of stimulation with EpCAM-transfected CHO cells armed either with bscAb EpCAM x CD3 alone, thus providing only a TCR-like primary signal (hatched columns), or with bscAb EpCAM x CD3 and 500 ng/ml heterodimeric B7-1 construct providing an additional costimulatory or second signal (black columns). Controls (empty columns): C1, purified naive CD8+ T cells incubated with 250 ng/ml bscAb EpCAM x CD3 and 500 ng/ml heterodimeric B7-1 construct in the absence of EpCAM-transfected CHO cells. C2, purified naive CD8+ T cells incubated with 250 ng/ml bscAb EpCAM x CD3 alone in the absence of EpCAM-transfected CHO cells. C3, unseparated PBMC incubated with 250 ng/ml bscAb EpCAM x CD3 and 500 ng/ml heterodimeric B7-1 construct in the presence of EpCAM-transfected CHO cells. C4, unseparated PBMC incubated with 250 ng/ml bscAb EpCAM x CD3 alone in the presence of EpCAM-transfected CHO cells. C5, unseparated PBMC incubated in the presence of EpCAM-transfected CHO cells without bscAb EpCAM x CD3 and heterodimeric B7-1 construct. The OD_{405} values for the TNF-alpha ELISA are shown.

In order to confirm that the switch in surface phenotype and the strong proliferative response actually indicate T cell priming as defined by effector functions, we investigated the secretion of TNF-alpha, which is typically produced by primed CD8+ T cells but not by their naive counterparts. As shown in Figure 11, only CD8+ T lymphocytes that underwent the CD45 phenotype switch exhibited strong TNF-alpha secretion. The definite proof that true priming of naive CD8+ T cells had occurred in the presence of bscAb EpCAM x CD3 and the B7-1 heterominibody was furnished in a cytotoxicity assay. Naive or *in vitro* primed T cells from the same donor were retargeted against EpCAM-positive Kato cells by the bispecific single-chain antibody (bscAb) EpCAM x CD3 (see Figure 1B). As shown in Figure 12, fresh purified naive CD8+ T cells showed almost no cytotoxic activity while *in vitro* primed CD8+ T lymphocytes proved to be highly cytotoxic, as did the PBMC that served as positive control. These results showed that the CD8+ T cells had become independent of a second costimulatory signal through prolonged prestimulation with bscAb EpCAM x CD3 and targeted B7-1, which is the main characteristic of T cell priming. In accordance with this observation, the presence of a costimulatory signal mediated by the EpCAM-specific B7-1 heterominibody during the cytotoxicity assay did not render naive CD8+ T cells cytotoxic, nor did it substantially enhance the cytotoxicity of PBMC or *in vitro* primed CD8+ T lymphocytes. As was shown for naive CD4+ T cells, the *in vitro* priming results obtained with CD8+ T lymphocytes costimulated via the B7-1 heterominibody could be reproduced with the B7-1 single-chain construct. Moreover, direct priming of naive

CD8+ T cells by non-APCs armed with bscAb EpCAM x CD3 and targeted B7 was still feasible in the confirmed absence of professional antigen presenting cells.

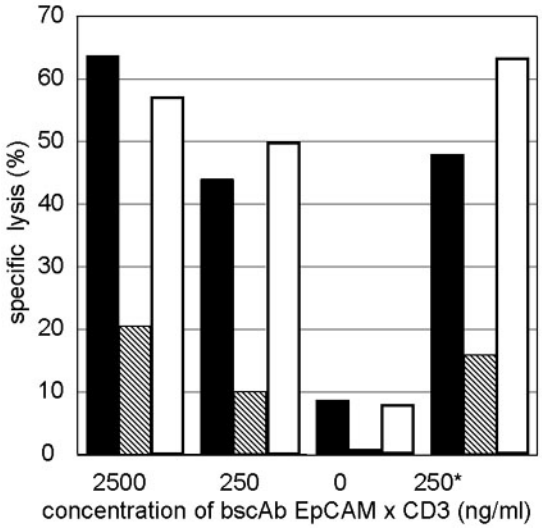


Figure 12. Cytotoxicity of different effector cell populations from the same donor redirected against EpCAM-positive Kato cells by the bispecific single-chain antibody (bscAb) EpCAM x CD3 as measured in a ^{51}Cr-release assay. Effector cells: unseparated PBMC (empty columns), purified naive CD8+ T cells (hatched columns) and CD8+ T cells primed for 6 days in the presence of 500 ng/ml heterodimeric B7-1 construct and 250 ng/ml bscAb EpCAM x CD3 (black columns). The concentration of bscAb EpCAM x CD3 during the ^{51}Cr-release assay is given in ng/ml. (*) indicates the presence of 500 ng/ml heterodimeric B7-1 construct during the ^{51}Cr-release assay.

Discussion

In the experiments presented, the change in CD45 isoform expression was used to monitor the priming of naive human T lymphocytes by tumor cells armed with targeted costimulatory molecules. By using purified naive CD45RA+ T cells exclusively, the appearance of CD45RO+ cells could clearly be attributed to the priming activity of the applied bifunctional constructs.

As expected, both naive CD4+ and CD8+ T cells showed only weak proliferation and failed to acquire the primed CD45 phenotype when receiving the primary activation signal from target cells armed with bscAb EpCAM x CD3 alone. In contrast, tumor cells armed with both bscAb EpCAM x CD3 and an EpCAM-specific B7-1 construct induced a complete switch in CD45 isoform to the RA-RO+ pattern of primed T cells, the transition being accompanied by a strong proliferative response. These results strongly suggested that B7-1 does not only contribute essentially to T cell activation but provides a second signal that is sufficient for T cell priming without further costimulatory requirements. To exclude that residual antigen presenting cells (APCs) such as dendritic cells were present in the T cell preparations and accounted for the observed priming effect, MHC II-positive cells were depleted to numbers below those capable of inducing an allogeneic MLR. In the depleted T cell preparations, tumor cells armed with bscAb EpCAM x CD3 and an EpCAM-specific B7-1 construct still induced the transition in CD45 isoform. Interestingly, the observed minimal modulation of CD45 isoform expression by

naive T lymphocytes stimulated with bscAb EpCAM x CD3 alone could be attributed to residual contaminating APCs as it was eliminated by depletion of MHC II-positive cells.

As additional evidence for true T cell priming, we investigated the gain of effector functions typical for primed T lymphocytes. Accordingly, the secretion of TNF-alpha, which is not found in naive CD8+ T cells (16,17), could only be demonstrated in CD8+ T lymphocytes that acquired the primed CD45 isoform pattern. Further functional evidence for CD8+ T cell priming came from the observed cytotoxic activity, as bscAb EpCAM x CD3 could redirect the cytotoxicity of primed CD8+ T cells against EpCAM-positive Kato cells whereas no cytotoxic activity was obtained with unprimed cells.

In the case of CD4+ T lymphocytes, priming was confirmed by assaying for the secretion of IFN-gamma, typical of T helper 1 (TH1) cells, and the TH2 cytokines IL-4 and IL-5, none of which are produced by naive CD4+ cells (18). Interestingly, costimulation through B7-1 led to strong secretion of IFN-gamma, while no IL-4 and IL-5 was detectable. Thus, naive CD4+ T cells primed in the presence of the exclusive costimulatory signal B7-1 could be shown to predominantly differentiate into TH1-cells. Virtually identical results were obtained with naive CD4+ T lymphocytes essentially free of contaminating MHC II-positive cells. In contrast to B7-mediated costimulation, targeted LFA-3, although inducing a strong T cell proliferation, did not provide naive CD4+ T lymphocytes with a second signal sufficient for T cell priming as indicated by CD45 isoform expression and cytokine secretion.

Several factors have been implicated in the regulation of naive T-cell differentiation into TH1 or TH2 effectors: (i) The intensity of the primary signal, (ii) the nature of the second signal, i.e. B7-1- or B7-2-mediated costimulation, (iii) the expression of further accessory molecules such as ICAM-1 or the OX40 ligand by APCs, and (iv) soluble differentiation factors such as IL-12 and IL-4.

It has been reported that a weak primary signal together with CD28-dependent costimulation induces TH2 differentiation (19). In our experiments however, priming of naive CD4+ T lymphocytes in the presence of B7-1-mediated costimulation generally led to TH1-cells. Particularly, no switch from TH1 to TH2 differentiation could be observed with decreasing strength of the primary signal provided by the stepwise reduction in bscAb EpCAM x CD3 concentration.

With murine T cells B7-1 and B7-2 costimulatory molecules have previously been found to distinctly trigger TH1 and TH2 differentiation respectively (20), while in our experiments an EpCAM-specific B7-2 construct as costimulatory signal clearly induced a TH1 cytokine pattern. As seen with B7-1, TH1/2-polarization triggered by B7-2 appeared to be independent of the strength of the primary signal (data not shown).

Besides the primary and the secondary T cell signals, a couple of "third signals", either mediated by further accessory molecules or by soluble differentiation factors, have been implicated in TH1/TH2-polarization. Salomon *et al*. (21) and Luksch *et al*. (22) reported that expression of ICAM-1 on antigen presenting cells inhibits the TH2 developmental pathway in the murine system. Accordingly, it has been speculated that ICAM-1 may favor TH1 differentiation of human CD4+ T cells as well. However, we could clearly demonstrate the development of TH1-cells although the tumor cell line used for stimulation of naive CD4+ T lymphocytes does not provide any autochthonous ICAM-like signals (23). Moreover, we could not observe any changes in TH1 polarization in the presence of targeted ICAM-1. Thus our results do not support the view that ICAM-1 exerts a major influence on TH1/TH2 differentiation.

According to the minimal signaling conditions that were applied, TH1 polarization does not seem to require additional signals. The absence of TH2 differentiation under minimal priming conditions indicates that human CD4+ T-lymphocytes absolutely require a "third signal" in order to follow the TH2 developmental pathway. The "third signal" may be mediated by antigen-related factors or by ligation of the OX40 antigen through APCs carrying the OX40 ligand, which has recently been shown to promote high levels of IL-4 production in naive human CD4+ T cells (24). IL-4 is known to be required as a soluble differentiation factor for the development of

TH2-cells, which themselves are a major source of IL-4 in a kind of positive feedback mechanism (25). Accordingly, addition of IL-4 induced naive CD4+ T cells to enter the TH2 developmental pathway under the described priming conditions. Another important soluble factor implicated in TH1 polarization is IL-12, which is produced by professional antigen presenting cells (26). However, in our experiments no traces of IL-12 were detected. Thus IL-12 proved to be dispensable for the development of TH1-cells, confirming the notion that the described minimal priming conditions (i.e. first and second signal only) were sufficient for TH1 differentiation.

Although several other groups reported on the targeting of B7 to the surface of tumor cells by using different recombinant constructs, the costimulatory activity of targeted B7 has not been probed by investigating its key function, that of providing the second signal during priming of naive T cells (5,6,7,8). In the majority of cases, the enhancement of proliferative T cell responses was reported, which is not by itself indicative of T cell priming as shown here with naive CD4+ T cells costimulated by LFA-3. In the publications cited above, the use of heterogeneous populations of T lymphocytes containing undefined numbers of primed cells precluded any statement as to whether priming was indeed achieved. Moreover, in three of these reports, T cells were prestimulated with PMA and IL-2 or with PMA alone; thus, the assessment of the effect by targeted B7 was further blurred.

The strategy to confer costimulatory capacity to tumor cells *in vivo* has been demonstrated by Townsend and Allison (27) to have a therapeutic effect. In a syngeneic mouse melanoma model they showed that B7-1 transfected melanoma cells induced both tumor rejection and T cell memory. Nevertheless, the transfer of this approach to human therapy still poses several problems. *Ex vivo* transduction of B7 into cells isolated from the primary tumor followed by reinjection as an autologous tumor vaccine is bound to remain an expensive and laborious individual therapy. Moreover, transfected cells prepared from the primary tumor may not cover the whole antigenic heterogeneity of the patient's tumor, particularly in view of systemically disseminated tumor cells and metastatic lesions. In contrast, B7 constructs specifically targeted to tumor cells *in vivo* by systemic application should be capable of exposing a representative antigenic spectrum of each patient's individual tumor to the immune system without requiring patient-specific manipulations. Finally, B7 targeting to the surface of tumor cells may not only enable direct priming of naive T-cells that specifically recognize processed tumor antigen with their T cell receptor, but may also enhance crosspresentation of tumor antigen by dendritic cells and subsequent crosspriming of tumor-specific T cells as observed with B7-transfected tumors in mice (4).

Abbreviations

bscAb, bispecific single-chain antibody; EpCAM, epithelial cell adhesion molecule

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Materials and methods

Construction and expression of a heterodimeric B7-1 antibody derivative

DNA fragments encoding the human Ig Ckappa and CH1gamma1 constant domains were obtained by PCR using the primer pairs 5'huCkBspEI and 3'huCKSalNot, and 5'huCkBspEI and 3'CH1huG1BspEI, respectively; two plasmids bearing the heavy and light chains of a human IgG1kappa antibody served as templates (28). The amplification products were cleaved with *Bsp*EI and *Sal*I (human Ckappa) or with *Bsp*EI alone (human CH1gamma1) and cloned into a derivative of Bluescript KS/M13+ vector designated BS-CTI. To this end BS-CTI was prepared by cleavage either with *Bsp*EI and *Sal*I (human Ckappa) or with *Bsp*EI and *Xma*I followed by dephosphorylation with calf intestinal phosphatase (human CH1gamma1). The BS-CTI vector was obtained by cloning the double stranded oligonucleotide CTI, obtained by annealing of 5'-phosphorylated single-stranded

oligonucleotides CTIsP and CTIasP, into the unique *Xba*I and *Sal*I restriction sites of Bluescript KS/M13+. By fusing the 3'*Bsp*EI site of the CH1gamma1-encoding fragment with the *Xma*I site of BS-CTI a polyhistidine tag was joined in frame to the C-terminus of the corresponding Ig domain. A DNA fragment comprising leader peptide and extracellular part of human B7-1 was obtained by RT-PCR from the total RNA of the human lymphoma cell line Raji using oligonucleotide primers 5'B7-1 and 3'B7-1. Subsequently, the PCR product was cloned *Eco*RI and *Bsp*EI into BS-CTI-CH1gamma1 thus completing the coding sequence of the B7-1 polypeptide arm, which was eventually subcloned *Eco*RI and *Sal*I into the mammalian expression vector pEF-ADA. As to the other polypeptide arm of the heterodimeric B7-1 construct, the EpCAM-specific M79 scFv fragment containing an Ig leader peptide but no Flag epitope was excised with *Eco*RI and *Bsp*EI from the corresponding version of the bispecific single-chain antibody 17-1A x CD3 (29) and accordingly subcloned into BS-CTI-Ckappa. Finally, the whole M79scFv/Ckappa polypeptide chain was subcloned *Eco*RI and *Sal*I into the mammalian expression vector pEF-DHFR.

Both polypeptide chains, B7-1/CH1gamma1 and M79scFv/Ckappa, were transfected by electroporation into Chinese hamster ovary (CHO) cells (ATCC CRL-9096) deficient in dihydrofolate reductase (DHFR), as described previously for the heavy and light chain of a human IgG1kappa antibody (28). Expression of the heterodimeric B7-1 construct was increased by gene amplification using the DHFR inhibitor methotrexate and the ADA inhibitor pentostatin as described. The transfectants were cloned by limiting dilution at final inhibitor concentrations of 500 nM methotrexate and 3 µM pentostatin. A high producer clone was identified by screening the culture supernatants by ELISA. A murine monoclonal antibody against human B7-1 was thus immobilized on a microtiter plate and bound B7-1 construct detected with a biotinylated polyclonal rabbit anti-human Ckappa antibody followed by peroxidase-conjugated avidin. For production of the B7-1 construct, the high producer clone was grown in roller bottles. The recombinant heterodimeric protein was purified from harvested supernatant in a three-step procedure comprising (i) cation exchange chromatography followed by (ii) a cobalt-chelate column that preferentially binds histidine-tagged proteins and (iii) gel filtration. Purification was monitored with the same ELISA used for the screening of culture supernatants. Purified B7-1 construct was analyzed under reducing and non-reducing conditions by SDS-PAGE in a 10% gel according to Laemmli (30), followed by protein staining with Coomassie brilliant blue R250.

ELISA with immobilized recombinant B7 ligands

The interaction of the B7-1 construct with CD28 and CTLA-4 was analyzed by ELISA. A microtiter plate was coated overnight at 4°C with 10 µg/ml recombinant truncated CD28 or CTLA-4-IgG fusion protein in PBS, respectively. Control proteins were immobilized at the same coating concentration. Coated wells were blocked with PBS containing 1% BSA for 1 h at room temperature. The purified B7-1 construct was subsequently diluted in PBS containing 1% BSA and incubated on immobilized B7 ligand for 1 h at room temperature. B7-1 construct bound to CTLA-4-IgG was detected with a biotinylated polyclonal rabbit anti-human Ckappa antibody (Pierce 31780, Perbio Science, Bonn, Germany) followed by peroxidase-conjugated avidin. B7-1 construct bound to truncated CD28 was detected with a polyclonal $F(ab')_2$-specific peroxidase-conjugated goat anti-human IgG antibody (Jackson ImmunoResearch 109-035-097, Dianova, Hamburg, Germany). The ELISA was developed with ABTS substrate solution (Roche, Mannheim, Germany) and the turnover of colored substrate measured at 405 nm.

CTLA-4-IgG fusion protein was obtained from R&D Systems (Wiesbaden, Germany). Truncated CD28 was produced as a soluble recombinant protein in CHO cells. Therefore, the DNA sequence encoding the extracellular part and the signal peptide of human CD28 was amplified by RT-PCR from the total RNA of PBMC using the oligonucleotide primers 5'hCD28-EcoRI and 3'hCD28-BspEI. In order to add a C-terminal polyhistidine tag, the PCR product was cleaved with *Eco*RI and *Bsp*EI and cloned into the vector BS-CTI cleaved with *Eco*RI and *Xma*I. Subsequently, the tagged CD28 fragment was subcloned *Eco*RI and *Sal*I into the expression vector pEF-DHFR and transfected into DHFR-deficient CHO cells. After stepwise gene amplification with a final MTX concentration of 500 nM, the protein was produced in roller bottles and purified from the culture supernatant as described for the heterodimeric B7-1 construct. Soluble truncated CD28 was detected by sandwich ELISA using

an immobilized monoclonal CD28 antibody (PharMingen 33741A, Heidelberg, Germany) and a peroxidase-conjugated monoclonal anti-histidine tag antibody (Roche, Mannheim, Germany).

Truncated human EGF receptor (EGFR) was produced as soluble recombinant protein in CHO cells as described for CD28, except that the primer pair 5'EGFR-XbaI and 3'EGFR-BspEI was used to amplify the extracellular part and signal peptide of human EGFR from the total RNA of the epidermoid carcinoma cell line A431 and *Eco*RI was replaced by *Xba*I in the cloning strategy. Soluble truncated EGFR was detected by sandwich ELISA using the immobilized monoclonal EGFR antibody 425 (31) and a peroxidase-conjugated monoclonal anti-histidine tag antibody (Roche, Mannheim, Germany). CD4-IgG fusion protein, which also served as negative control, was produced as described (32) except that the amplifiable expression vector pEF-DHFR and DHFR-deficient CHO cells were used.

Costimulatory single-chain constructs

For the construction of targeted costimulatory single-chain proteins, the EpCAM-specific murine scFv antibody fragment 4-7 was used instead of the monoclonal anti-EpCAM antibody M79, because the corresponding scFv fragment lost antigen binding when it was joined through a flexible Gly_4Ser_1 linker with the C-terminus of the extracellular part of human B7-1 (data not shown). The B7-1/4-7 single-chain construct was produced as described (33). Briefly, the 5'*Eco*RI and 3'*Bsp*EI DNA fragment comprising leader peptide and extracellular part of human B7-1 was fused with a 5'*Bsp*EI and 3'*Sal*I DNA fragment comprising, from 5' to 3', a Gly_4Ser_1 linker, the EpCAM-specific scFv fragment 4-7 as well as a His_6 tag and the fusion product cloned *Eco*RI and *Sal*I into the pEF-DHFR expression vector. The ICAM-1/4-7 single-chain protein was constructed by PCR amplification of a DNA fragment comprising leader peptide and extracellular part of human ICAM-1 from the cDNA of HL-60 cells using oligonucleotide primers 5'ICAM-1 and 3'ICAM-1, followed by replacement of the *Eco*RI-*Bsp*EI fragment encoding B7-1 by the *Eco*RI-*Bsp*EI ICAM-1 fragment in the corresponding pEF-DHFR expression plasmid. To construct the B7-2/4-7 single-chain protein, the scFv fragment 4-7 from pEF-DHFR containing the B7-1/4-7 construct was subcloned *Bsp*EI and *Sal*I into BS-CTI. Subsequently, a DNA fragment comprising leader peptide and extracellular part of human B7-2 was amplified by PCR from the cDNA of Raji cells using oligonucleotide primers 5'B7-2 and 3'B7-2 and cloned *Xba*I and *Bsp*EI into BS-CTI-4-7. Finally the B7-2/4-7 construct was subcloned *Xba*I and *Sal*I into pEF-DHFR. Another DNA fragment comprising leader peptide and extracellular part of human LFA-3 was amplified by PCR from the cDNA of U937 cells using oligonucleotide primers 5'LFA-3 and 3'LFA-3 and the EpCAM-specific LFA-3/4-7 single-chain protein constructed like B7-2/4-7. The costimulatory single-chain proteins were transfected into DHFR-deficient CHO cells. Gene amplification, as well as protein production and purification, were carried out as described above for soluble CD28. The costimulatory single-chain constructs were detected by four different sandwich ELISAs using immobilized monoclonal antibodies against human B7-1, B7-2, LFA-3 or ICAM-1 and a peroxidase-conjugated monoclonal anti-histidine tag antibody (Roche, Mannheim, Germany). The antigen specificity of the costimulatory single-chain constructs was confirmed by ELISA with immobilized recombinant EpCAM antigen (34); EpCAM-bound constructs were detected with a peroxidase-conjugated monoclonal anti-histidine tag antibody (Roche, Mannheim, Germany).

Flow cytometry

To investigate the specific interaction of the heterodimeric B7-1 construct with the human EpCAM antigen, 2 x 10^5 EpCAM-transfected CHO cells were incubated with 4 µg/ml purified B7-1 heterominibody for 30 minutes on ice, followed by three washing steps in PBS. Recombinant B7-1 construct bound to the cells was detected with a FITC-conjugated polyclonal goat anti-human Ckappa antibody (Coulter 6604287, Krefeld, Germany). Flow cytometry was performed on a FACScan® (Becton Dickinson, Heidelberg, Germany).

Flow cytometric analysis of CD45 isoform expression was carried out by double staining of 1 x 10^5 cells with a PE-conjugated monoclonal anti-CD45RA antibody (Coulter, 2H4, 6603904, Krefeld, Germany) and a FITC-conjugated monoclonal anti-CD45RO antibody (DAKO, UCHL-1, F 0800, Hamburg, Germany) for 30 minutes on

ice. Flow cytometric monitoring of T cell purification was equally carried out by single stainings with a Tricolor-conjugated monoclonal anti-CD4 antibody (MHCD0406,), a Tricolor-conjugated monoclonal anti-CD8 antibody (MHC0806) and a FITC-conjugated monoclonal anti-CD28 antibody (MHCD2801), all from Medac (Hamburg, Germany).

Purification of naive T cells

Mononuclear cells (PBMC) were prepared by Ficoll density centrifugation from 500 ml peripheral blood obtained from a healthy donor. CD4+ and CD8+ T cells were isolated by negative selection using commercially available cell separation kits (R&D Systems, HCD4C-1000 and HCD8C-1000 respectively, Wiesbaden, Germany). Each CD4+ or CD8+ T cell column was loaded with 2 x 10^8 PBMC which had been preincubated with the manufacturer's antibody cocktail, except that the CD8+ T cell cocktail was supplemented with 1 µg monoclonal anti-CD11b antibody (Coulter 0190, Krefeld, Germany) per column. Successful separation of CD4+ and CD8+ T cells was controlled by flow cytometry after single staining with an anti-CD4 or anti-CD8 antibody respectively. Absence of CD11b+ cells from CD8+ T cell preparations was confirmed by single staining with an anti-CD28 antibody, since CD11b+ CD8+ T cells are known to be CD28- and vice versa.

CD45RO+ cells were removed from purified CD4+ or CD8+ T cells by incubation with a murine monoclonal anti-CD45RO antibody (PharMingen, UCHL-1, 31301, Heidelberg, Germany), followed by separation using magnetic beads conjugated with a polyclonal sheep anti-mouse Ig antibody (Dynal, 110.01, Hamburg, Germany). For depletion of residual antigen presenting cells (e.g. dendritic cells) purified CD4+ or CD8+ T cells were coincubated with murine monoclonal antibodies against CD45RO and HLA-DR, DP, DQ (PharMingen, 32381A, Heidelberg, Germany) prior to incubation with magnetic anti-mouse Ig beads. The purity of the remaining naive CD4+ or CD8+ T cells proved to be 95 to 97% as determined by flow cytometry after double staining with anti-CD45RA and anti-CD45RO. The yields of naive T cells were 2 to 3 x 10^7 (CD4) and 5 x 10^6 (CD8) per 500 ml peripheral blood.

Allogeneic mixed lymphocyte reactions (MLRs)

Cells obtained from different steps of the naive CD4+ or CD8+ T lymphocyte purification protocol above were used as stimulator cells, i.e. PBMC as well as CD4+ or CD8+ T lymphocytes prior to and after CD45RO/MHC II depletion. Bulk CD4+ T cells of another healthy donor purified using a commercially available cell separation kit (R&D Systems, HCD4C-1000, Wiesbaden, Germany) served as responder cells. The stimulator cells were irradiated with 2000 rad and seeded into 96-well round-bottom microtiter plates at 1 x 10^6 cells per well. Subsequently, 5 x 10^5 responder cells per well were added at a final volume of 200 µl RPMI 1640 culture medium supplemented with 10% human AB serum, 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, 10 mM HEPES buffer, 1x non-essential amino acids (Gibco, Life Technologies, Karlsruhe, Germany) and 50 µM beta-mercaptoethanol. The cell mixture was cultured for 4 days at 37°C and 6% CO_2. In order to measure responder cell proliferation, the thymidine analogue BrdU was added on day 3 followed by quantitation of incorporated BrdU after a further 16 hours of culture using an ELISA-based cell proliferation kit (Roche, BrdU colorimetric, 1647229, Mannheim, Germany) according to the manufacturer's protocol. For the BrdU-ELISA cells were transferred from round- to flat-bottom 96-well microtiter plates.

***In vitro* priming of purified naive T cells**

EpCAM-transfected CHO cells (2.5 x 10^4 per well) were incubated in a 96-well flat-bottom culture plate for 2 hours. After the cells had adhered to the plastic, they were irradiated with 14000 rad. Subsequently, 5 x 10^4 purified naive T cells per well were added in RPMI 1640 medium supplemented with 10% human AB serum, 100 U/ml penicillin, 100 mg/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, 10mM HEPES buffer, 1x non-essential amino acids (Gibco, Life Technologies, Karlsruhe, Germany) and 50 µM beta-mercaptoethanol. The bispecific single-chain antibody (bscAb) EpCAM x CD3 (29,34) was added alone at concentrations ranging from

2 to 250 ng/ml or together with 500 ng/ml EpCAM-specific B7-1 heterominibody. As controls, bscAb EpCAM x CD3 was omitted or naive T cells were incubated without CHO cells in the presence of 250 ng/ml bscAb EpCAM x CD3 with and without 500 ng/ml B7-1 heterominibody. Unseparated PBMC incubated with EpCAM-transfected CHO cells in the presence of 250 ng/ml bscAb EpCAM x CD3, with and without 500 ng/ml B7-1 heterominibody, as well as in the absence of both bscAb EpCAM x CD3 and B7-1 heterominibody, served as further controls. A heterominibody concentration of 500 ng/ml was chosen as the maximum concentration that by itself did not affect CD45 isoform expression with both naive CD4+ and CD8+ T cells. All experiments were carried out in triplicate wells. Furthermore, a set of three identical 96-well plates was prepared in order to make sure that sufficient cells were available for flow cytometry and proliferation analysis. On day 4, cells from one 96-well plate were harvested and subjected to flow cytometric analysis of CD45 isoform expression; the corresponding culture supernatants were stored at -20°C for later cytokine analysis. In order to measure T cell proliferation, the thymidine analogue BrdU was added to the cells of another 96-well plate on day 3 followed by quantitation of incorporated BrdU on day 4 using an ELISA-based cell proliferation kit (Roche, BrdU colorimetric, 1647229, Mannheim, Germany) according to the manufacturer's protocol. On day 4, half of the supernatant was removed from each well of the third 96-well plate and replaced by fresh medium adjusted to the corresponding concentrations of bscAb EpCAM x CD3 and/or B7-1 heterominibody. On day 6, the cells of this 96-well plate were harvested and their CD45 isoform expression pattern was analyzed by flow cytometry. Simultaneously, the corresponding supernatants from days 4 and 6 were subjected to cytokine analysis. For T cell cultures extending beyond day 6, the cells were transferred into a new microtiter plate on day 7 without the addition of fresh CHO cells, and half of the supernatant was removed and replaced by fresh medium adjusted to the corresponding concentrations of bscAb EpCAM x CD3 and/or B7-1 construct.

Cytokine secretion was investigated with commercially available ELISA kits (PharMingen, Heidelberg, Germany) measuring the concentrations of TNF-alpha (2600KK), IFN-gamma (2677KK), IL-4 (2678KK), IL-5 (2679KK) and IL-12 (2621K1). Cells and supernatants from three identical wells (triplicates) were pooled for flow cytometry and cytokine analysis. For the proliferation assay, the incorporation of BrdU was measured for each of the three wells and the average calculated.

In vitro priming experiments with costimulatory single-chain constructs were carried out as described above, except that the B7-1 heterominibody was replaced either by 500 ng/ml of the EpCAM-specific B7-1, B7-2 or LFA-3 single-chain construct or by 125 ng/ml B7-1 single-chain construct and 500 ng/ml of the EpCAM-specific ICAM-1 single-chain construct. The costimulatory single-chain constructs were used at concentrations that by themselves did not affect CD45 isoform expression of naive CD4+ T cells. Cell culture was carried out at 37°C and 6% CO_2 for all priming experiments.

Cytotoxicity assay

The ^{51}Cr-release assay measuring T cell cytotoxicity redirected against EpCAM-positive Kato (ATCC HTB-103) cells by bscAb EpCAM x CD3 was carried out as described previously (34). CD8+ T cells that had been primed for 6 days in the presence of B7-1 heterominibody and 250 ng/ml bscAb EpCAM x CD3 were used as effector cells. Fresh naive CD8+ T cells isolated from the same donor and unstimulated PBMC served as negative and positive controls respectively.

Briefly, for each well in a round-bottom microtiter plate, 2 x 10^5 effector cells were mixed with 2 x 10^4 ^{51}Cr-labeled Kato cells and incubated for 18 h in the presence of either 250 ng/ml or 2500 ng/ml bscAb EpCAM x CD3, or in the absence of the bispecific antibody. The induction of T cell cytotoxicity by 250 ng/ml bscAb EpCAM x CD3 was also assessed in the presence of 500 ng/ml EpCAM-specific B7-1 heterominibody.

Maximum ^{51}Cr-release was determined by lysis of target cells with Maly buffer (2% SDS, 0.37% EDTA, 0.53% Na_2CO_3). The spontaneous ^{51}Cr-release was determined with target cells incubated in the absence of effector cells and bispecific antibody. Specific lysis was calculated as [(cpm, experimental release) - (cpm, spontaneous release)] / [(cpm, maximal release) - (cpm, spontaneous release)]. The cytotoxicity assay was carried out with triplicate samples.

Primers

CTIsP: 5'-CTA GAA TTC TTC GAA TCC GGA GGT GGT GGA TCC GAT ATC CCC GGG CAT CAT CAC CAT CAT CAT TGA G-3'
CTIasP: 5'-TCG ACT CAA TGA TGA TGG TGA TGA TGC CCG GGG ATA TCG GAT CCA CCA CCT CCG GAT TCG AAG AAT T-3'
5'CH1huG1BspE1: 5'-AAT TCC GGA ACC CCG CTG GGT GAC ACC ACC CAC ACC GCT AGC ACC AAG GGC CCA TCG GTC TTC C-3'
3'CH1huG1BspE1: 5'-AAT TCC GGA ACT AGT TTT GTC ACA AGA TTT GG-3'
5'HuCKBspE1: 5'-AAT TCC GGA ACC CCG CTG GGT GAC ACC ACC CAC ACC CGT ACG GTG GCT GCA CCA TCT GTC TTC-3'
3'HuCKSalNot: 5'-ATA AGA ATG CGG CCG CGT CGA CTA ACA CTC TCC CCT GTT GAA GCT C-3'
5'B7-1: 5'-GCA GAA TTC ACC ATG GGC CAC ACA CGG AGG CAG-3'
3'B7-1: 5'-TGG TCC GGA GTT ATC AGG AAA ATG CTC TTG CTT G-3'
5'B7-2: 5'-AAG TCT AGA AAA TGG ATC CCC AGT GCA CTA TG-3'
3'B7-2: 5'-AAT TCC GGA TGG GGG AGG CTG AGG GTC CTC AAG C-3'
5'ICAM-1: 5'-CTC GAA TTC ACT ATG GCT CCC AGC AGC CCC CG-3'
3'ICAM-1: 5'-GAT TCC GGA CTC ATA CCG GGG GGA GAG CAC-3'
5'LFA-3: 5'-AAT CTA GAA CCA TGG TTG CTG GGA GCG ACG-3'
3'LFA-3: 5'-AAG TCC GGA TCT GTG TCT TGA ATG ACC GCT GC-3'
5'hCD28-EcoRI: 5'-CGG AAT TCC ACC ATG CTC AGG CTG CTC TTG GC-3'
3'hCD28-BspEI: 5'-GGA TCC GGA GGG CTT AGA AGG TCC GGG AAA TAG-3'
5'EGFR-XbaI: 5'-GGT CTA GAC GAT GCG ACC CTC CGG GAC G-3'
3'EGFR-BspEI: 5'-GGC TCC GGA CGG GAT CTT AGG CCC ATT C-3'

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