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(54) Title: PHENOTYPIC KNOCKOUT OF CELL-SURFACE PROTEINS

(57) Abstract: The invention exploits the finding that the activity of a co-stimulatory ligand in an antigen presenting cell (APC) can be blocked by expressing a receptor for the ligand in the APC such that it is targeted to the endoplasmic reticulum (ER), where it binds to the ligand in the ER lumen and retains the ligand there. The invention thus relates to a protein comprising: (a) a first sequence which causes the protein to be retained in the endoplasmic reticulum; and (b) a second sequence which can bind to a co-stimulatory ligand of an antigen presenting cell.



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PHENOTYPIC KNOCKOUT OF CELL-SURFACE PROTEINS

All documents cited herein are incorporated by reference in their entirety.

TECHNICAL FIELD

This invention is in the field of T-cell immunology.

5 BACKGROUND ART

Immunosuppression by inhibiting T-cell responses has various applications [1]. T-cell activation requires two separate signals, and delivery of signal 1 alone induces a refractory state (“anergy”), defined as the inability to produce IL-2 after subsequent antigenic exposure. For full activation to occur, the cell must also receive signal 2. Signal 1 is usually provided by the interaction of the
10 TCR/CD4 complex with either allogeneic MHC or antigenic peptide complexed with self MHC. Signal 2 is supplied by the interaction between B7 molecules (B7-1 and B7-2, also known as CD80 and CD86, respectively) on the antigen-presenting cell (APC) and CD28 on the T-cell

Much attention has focused on preventing delivery of this second signal to the T-cell, thereby rendering them anergic. The “co-stimulation blockers” include CTLA4-Ig [2], LEA29Y, and antibodies against
15 CD40, CD40L, CD154, CD28, or B7. These co-stimulation blockers have received attention for enhancing transgene expression [3] and for treating diseases such as lupus [4], psoriasis vulgaris [5], asthma [5], rheumatoid arthritis [6] and transplant rejection [7,8].

In an alternative strategy, dendritic cells are treated with a variety of agents (such as dexamethasone) in order to “freeze” them in an immature phenotype where they express low levels of CD80/86 and so
20 induce anergy in antigen specific T-cells [9,10]. It has been shown *in vitro* and *in vivo* that anergic cells can act as regulatory cells, and so tolerance induced to a limited number of epitope can spread to regulate immunity against other determinants expressed by tissues [11,12]. It has been proposed that administration of immature dendritic cells (DCs) expressing appropriate antigens could be used to prevent transplant rejection and treat autoimmune diseases [13]. However, these DCs may revert to an
25 activated phenotype *in vivo*, especially in sites with high concentrations of inflammatory cytokines.

It is an object of the invention to provide further and improved ways of blocking co-stimulation of T-cells, and in particular for blocking the interaction of co-stimulatory proteins. More generally, it is an object of the invention to provide further and improved ways of altering the cell-surface phenotype of T-cells (and other cells) *e.g.* in order to modify their immunogenic properties.

30 DISCLOSURE OF THE INVENTION

It has surprisingly been found that the activity of a co-stimulatory ligand in an antigen presenting cell (*e.g.* the ligand B7) can be blocked by expressing a receptor for the ligand (*e.g.* the CTLA4 receptor for B7) in the APC such that it is targeted to the endoplasmic reticulum (ER), where it binds to the ligand in the ER lumen and retains the ligand there. Rather than prevent transcription and translation
35 of the co-stimulatory ligand in the antigen presenting cell, therefore, the invention sequesters a

receptor for the ligand in the ER which in turn sequesters the ligand in the ER, thereby inhibiting the ligand's display on the APC surface. Thus co-stimulatory ligands can be knocked out at a phenotypic level. Furthermore, compared to administration of soluble proteins, the invention offers the advantage of being specific to antigens expressed by the APCs, thereby avoiding generalised immunosuppression.

The invention therefore provides a protein comprising: (a) a first sequence which causes the protein to be retained in the endoplasmic reticulum; and (b) a second sequence which can bind to a co-stimulatory ligand of an antigen presenting cell.

In one embodiment, the protein comprises a receptor for the B7 ligand (*e.g.* CTLA4) to the C-terminus of which an ER-retention signal sequence (*e.g.* KDEL) has been fused. While the use of CTLA4 makes the invention superficially similar to the known CTLA4-Ig approach to blocking B7 activity, a basic difference is that the invention uses CTLA4 to inhibit B7 from being displayed on the cell surface, rather than using it to block B7 once it has already been displayed. Expressing a T-cell surface receptor within an antigen presenting cell is thus fundamentally different from the CTLA4-Ig approach.

More generally, the invention is not limited to receptors which bind to co-stimulatory ligands. Rather, the same approach can be used to target any cell surface receptor to the ER and thereby sequester that receptor's ligand. Thus the invention provides a protein comprising: (a) a first sequence which causes the protein to be retained in the endoplasmic reticulum; and (b) a second sequence from a cell surface receptor. The receptor of the second sequence is normally expressed at the cell surface, but the first sequence instead retains the receptor's ligand-binding activity in the ER, where it can bind its ligand to inhibit the ligand's exit from the ER. This approach is particularly suitable for inhibiting expression of ligands which would normally be displayed on the cell surface *i.e.* the receptor and the ligand would normally be displayed on the surface of different cells such that they could interact, but the invention moves this interaction into the ER. Thus the invention provides a technique for inhibiting cellular interactions which are mediated via surface-located proteins.

The invention also provides a method for inhibiting the cell-surface expression of a first protein in a cell of interest by expressing in the cell of interest a second protein comprising: (a) a first sequence which causes the second protein to be retained in the endoplasmic reticulum; and (b) a second sequence which can bind to the first protein.

The first sequence

The protein of the invention includes a first sequence which causes the protein to be retained in the endoplasmic reticulum. Retention of proteins in the ER lumen has been widely studied [14,15] and the optimum amino acid sequence for retention in animals is KDEL, which is preferred for use with the invention. Other sequences can also function (*e.g.* HDEL, DDEL, ADEL, SDEL, QDEL, KEDL, KDEI, *etc.*), and different organisms have different preferences for these varied sequences. The

consensus pattern using PROSITE notation is [KRHQSA]-[DENQ]-E-L. Liver esterases are also believed to use the sequences HTEL, HVEL and HIEL for ER retention, and C-terminal dilysine sequences of general formula KKXX are able to re-direct transmembrane proteins to the ER [15].

5 The introduction of retention sequences to sequester proteins in the ER has previously been used for antibodies ("intrabodies" [16-22]) and chemokines ("intrakines" [23-29]). It has also been used to sequester other normally-secreted proteins such as erythropoietin [30].

Proteins of the invention may also have a signal sequence for targeting to the ER, at least in their nascent forms. In mature form, such signal sequences will typically have been cleaved in the ER lumen, and are not required once the protein has reached the ER. Where the protein of the invention
10 enters a cell (*e.g.* by endocytosis or phagocytosis), therefore, rather than being synthesised within a cell (*i.e.* by translation), then a signal sequence is not required.

An ER targeting sequence will generally be located at or near the N-terminus of the protein, whereas an ER retention sequence will be located at the C-terminus.

The second sequence

15 The protein of the invention includes a second sequence. In preferred embodiments of the invention, the second sequence can bind to a co-stimulatory ligand of an antigen presenting cell. The second sequence will generally be derived from a natural receptor for the co-stimulatory ligand. Details of various co-stimulatory ligands and receptors can be found in references 31 to 33.

The co-stimulatory ligand is preferably B7, and the second sequence may bind to B7-1 (CD80)
20 and/or to B7-2 (CD86). B7-1 and B7-2 are both present on the surface of antigen presenting cells. They are type I proteins in the immunoglobulin superfamily. B7-1 expression is found on activated B-cells, activated T-cells, activated dendritic cells and macrophages. B7-2 is constitutively expressed on interdigitating dendritic cells, Langerhans cells, peripheral blood dendritic cells, memory B-cells and germinal center B-cells. Additionally, B7-2 is expressed at low levels on monocytes and is up-
25 regulated through IFN- γ stimulation. Human B7-1 and B7-2 share approximately 26% amino acid identity; mouse B7-1 and B7-2 share approximately 28% aa identity. Human and mouse B7-1 share approximately 44% aa identity. Human and mouse B7-2 share approximately 50% aa identity.

Both B7-1 and B7-2 are capable of binding receptors CD28 and CTLA4. Binding to CTLA4 has been shown to have a 20-100-fold higher affinity than binding to CD28. Both human and mouse
30 B7-1 and B7-2 can bind to either human or mouse CD28 and CTLA4. CD28 plays a role in activation, whereas CTLA4 functions as an inhibitory receptor important for down-modulating the immune response. B7-2 is generally the first B7 molecule encountered, due to its constitutive expression on numerous APCs. There are some differences in the functions of B7-1 and B7-2 but, in the main, the use of B7-1 or B7-2 depends on the type of APC encountered and its activation state.

Where the second sequence in the protein of the invention binds to B7, it is preferably derived from a natural receptor for B7. Such receptors include CTLA4 (CD152) and CD28. Like B7, CD28 and CTLA4 are also members of the Ig superfamily. CD28 is expressed on nearly all CD4+ T-cells and about half of CD8+ T-cells, and is also expressed on developing thymocytes. CTLA4 is not
 5 constitutively expressed; rather, it is rapidly up-regulated after CD28 ligation and T-cell activation. The genes encoding CD28 and CTLA4 are closely linked on human chromosome 2 and mouse chromosome 1. Human and mouse CD28 share approximately 68% aa identity. Human and mouse CTLA4 share approximately 76% aa identity. CD28 and CTLA4 share approximately 30% aa identity. Because of its higher affinity for B7, the invention uses CTLA4 in preference to CD28.

10 Other than B7-1 and B7-2, co-stimulatory ligands from APCs which may be bound by the second sequence include:

- B7-H1 (also known as PD-L1, for “programmed death ligand 1”). The natural receptor for the B7-H1 ligand is PD-1 (“programmed death-1”). B7-H1 shares approximately 20% aa identity with B7-1 and 15% aa identity with B7-2. Human and mouse B7-H1 share approximately 69% aa
 15 identity. B7-H1 and PD-L2 (see below) share approximately 41% aa identity. B7-H1 is widely expressed on normal tissues (*e.g.* liver, lung, pancreas, heart), but not on resting peripheral blood cells. Expression on monocytes and dendritic cells is up-regulated with IFN- γ stimulation or upon activation. B7-H1 does not bind to CD28, CTLA-4 or ICOS.
- PD-L2 (“programmed death ligand 1”; also known as B7-DC). The natural receptor for the
 20 PD-L2 ligand is PD-1. Human and mouse PD-L2 share approximately 72% aa identity. PD-L2 and B7-H1 (see above) share approximately 41% aa identity. PD-L2 is widely expressed on normal tissues (*e.g.* liver, lung, pancreas, heart), but not on resting peripheral blood cells. Expression on monocytes and dendritic cells is up-regulated with IFN- γ stimulation or upon activation. PD-L2 does not bind to CD28, CTLA-4 or ICOS.
- 25 – B7-H2 (also known as B7h, B7RP-1, GL50, ICOSL, LICOS). The natural receptor for the B7-H2 ligand is ICOS. B7-H2 is a type I protein in the Ig superfamily. Human and mouse B7-H2 share approximately 49% aa identity. B7-H2 is expressed constitutively on resting B cells and dendritic cells, and at low levels on monocytes. IFN- γ stimulation up-regulates expression on these cell types. B7-H2 is the ligand for ICOS and does not bind to CD28, CTLA-4 or PD-1.
- 30 – B7-H3. B7-H3 is a type I Ig superfamily transmembrane protein. B7-H3 shares approximately 24% sequence identity with B7-1, 26% with B7-2, 28% with B7-H1, 29% with PD-L2 and 29% with B7-H2. Human and mouse B7-H3 share 88% aa identity. There are two isoforms of the human B7-H3 protein which differ in the N-terminal sequence, exhibit some aa substitutions and, most importantly, differ in the number of extracellular Ig domains. The isoform with four, rather
 35 than two, Ig domains is most widely expressed in human tissues and has been designated as B7-H3b. This form appears to be the result of a gene duplication event that occurred in humans.

The mouse homolog has two extracellular Ig domains, as is usual for B7 family members. B7-H3 expression is not found on resting peripheral blood cells or dendritic cells. Expression on these cells, however, can be up-regulated by cytokine exposure or a phorbol myristate acetate (PMA)/ionomycin combination. B7-H3 is widely expressed in other tissues. B7-H3 does not bind CD28, CTLA-4, ICOS or PD-1. A recombinant B7-H3/Ig fusion protein can bind activated T cells, suggesting that a counter-receptor is present on activated T cells. Data obtained with the fusion protein demonstrate that B7-H3 mediates T cell proliferation and IFN- γ production.

In addition to CTLA4 and CD28, therefore, natural receptors from T-cell which can be used for deriving the second sequence include:

- 10 – ICOS (“inducible costimulator”; also known as AILIM (“activation-inducible lymphocyte immunomodulatory molecule”), CRP-1 (“CD28-related protein-1”), H4). ICOS shares ~39% aa similarity with CD28 and CTLA-4. Human and mouse ICOS share approximately 72% aa identity. B7-H2 is the ligand for ICOS. ICOS is not expressed on naïve T cells. ICOS expression is rapidly up-regulated, however, after TCR ligation. ICOS is expressed on most CD45RO⁺ cells. Differential expression of ICOS on Th1 vs. Th2 cells suggests that it may also play a role in Th cell differentiation. In contrast to CD28, ICOS may be more important in regulating cytokine production in effector T-cells and recently activated T-cells. Further, ICOS and CD28 stimulation differ in the pattern of cytokines up-regulated.

Thus ICOS may be expressed in an APC with an ER-retention sequence in order to sequester B7-H2 in the ER, thereby promoting anergy in T-cells which are stimulated by such APCs.

- PD-1. PD-1 is a member of the CD28/CTLA4 family of Ig co-stimulatory immunoreceptors. PD-1 is most closely related to CTLA4, sharing approximately 24% aa identity. Human and mouse PD-1 share approximately 63% aa identity in the extracellular domains. PD-1 expression is not found on un-stimulated T-cells, B-cells or myeloid cells. After activation, however, PD-1 expression is up-regulated on these cells. B7-H1 and PD-L2 are ligands for PD-1. Similar to CTLA4, PD-1 appears to transmit a negative immunomodulatory signal. When PD-1 binds B7-H1 or PD-L2, TCR-mediated proliferation is inhibited and cytokines are produced. B7-H1/PD-L2/PD-1 interactions have been implicated in negative regulation of some immune responses and may play an important role in the regulation of peripheral tolerance.

Thus PD-1 may be expressed in an APC with an ER-retention sequence in order to sequester PD-L1 and/or PD-L2 in the ER. As co-stimulation of T-cells by these two ligands has an immunosuppressive effect then inhibition of their cell-surface display will be immunostimulatory for T-cells which bind such APCs. Stimulating immune responses in this way is useful in various situations *e.g.* in cancer therapy or in immunosuppressed patients.

Rather than use a full-length natural receptor, the invention will typically use a portion of the receptor which retains affinity for the ligand. This is important when using sequences such as KDEL

with receptors which are naturally membrane proteins, as these sequences do not mediate ER-retention of membrane proteins. For example, CTLA4 has been dissected [34] and its B7-binding region has been separated from the rest of the protein. In CTLA4-Ig, for instance, the extracellular domain of CTLA4 is fused to an Ig-C γ 1 chain [35]; the C-terminal transmembrane and cytoplasmic portions of the protein are not required for B7 binding (and deletion of the C-terminal tail gives better results according to the invention). PD-1 has been similarly dissected. The skilled person is thus readily able to derive B7-binding sequences from known CTLA4 and CD28 sequences. More generally, the skilled person can divide the amino acid sequence of cell surface receptors into extracellular ligand-binding regions, transmembrane regions and cytoplasmic signalling regions based on changes in local amino acid composition *e.g.* transmembrane regions feature hydrophobic amino acids and have a characteristic length. Algorithms are available for predicting where these regions are located for any given amino acid sequence.

In general, the second sequence will comprise: (a) a sequence with at least 50% (*e.g.* 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or more) amino acid sequence identity to the ligand-binding region of the receptor; and/or (b) a fragment of at least 7 (*e.g.* 8, 9, 10, 12, 14, 16, 18, 20, 25, 30, 35, 40, 50, 75, 100, or more) contiguous amino acids from the ligand-binding region of the receptor. The second sequence preferably includes any amino acid motif from the receptor which is essential for ligand binding. The ligand-binding region will generally be from the extracellular portion of the receptor, which is normally in the N-terminus part of the mature protein.

For example, where the receptor is human CTLA4, the ligand binding region is amino acids 38-161 (the extracellular domain) of the amino acid sequence given in GenBank entry NP_005205.2 (GI:21361212) [SEQ ID NO: 14 herein], and the second sequence preferably includes the hexapeptide MYPPPY [SEQ ID NO: 15].

Although ligands from one organism (*e.g.* pig B7) are able to bind to receptors from another organism (*e.g.* human CTLA4), the highest avidity is generally seen with proteins from the same organism. Where the second sequence is derived from a natural receptor, therefore, it is preferred that it should be derived from the same organism (*e.g.* human) for which a therapeutic effect is desired. This will generally be the organism to which it is intended to administer the protein of the invention (*e.g.* from human CTLA4), but if the intention is to suppress anti-xenograft T-cells then the receptor may be from the xenogeneic organism [7].

As an alternative to using natural receptors for co-stimulatory ligands (*e.g.* CTLA4, CD28, PD1, ICOS) the invention can utilise an anti-ligand antibody, although receptors such as CTLA4 are preferred because they can block expression of all their ligands (*e.g.* CD80 and CD86), rather than needing a separate antibody for each target, because they have a lower inherent immunogenicity, and because correct folding is typically easier. Monoclonal antibodies can easily be produced against ligands of interest and the variable domains of such antibodies can be used as second sequences for the invention. As antibodies naturally have two separate chains, however, it is preferred to use a

single chain antibody (*e.g.* a scFv) in which the light and heavy chain variable domains are joined by a linker to give a single polypeptide chain. Kits for preparing scFv's are available off-the-shelf, and anti-ligand scFvs are preferred second sequences for use with the invention. Single domain antibodies can also be obtained from camelids or sharks [36], or by camelisation [37].

- 5 Anti-ligand antibodies can be prepared by immunisation, or they can be prepared by screening libraries *e.g.* phage display libraries [38], or other display systems [39]. Display can also be used to select non-antibody peptides with affinity for co-stimulatory ligands, for use as second sequences.

Techniques for retaining antibodies in the ER are well-known, having been established for intracellular antibodies ("intrabodies") [16-22] *e.g.* direct fusion to the C-terminus.

- 10 As a further alternative to using natural receptors, the invention can utilise artificial receptors. These can be identified by techniques such as phage display or directed evolution, using a selection assay which identifies proteins which retain the ability to bind specifically to a ligand of interest. For example, reference 40 describes the use of phage display to identify peptide mimics of the CTLA4 binding domain, and reference 41 describes phage display for identifying peptide motifs that bind to
15 a B7-1 monoclonal antibody.

It will be appreciated that, although the description refers to "a second sequence" in the protein of the invention which can bind to the co-stimulatory ligand, the amino acids within the protein which give rise to the ligand-binding activity need not be continuous. For example, while the extracellular domain of CTLA4 is an uninterrupted fragment of CTLA4 which provides B7 affinity in a
20 contiguous sequence of amino acids, therefore, that contiguous sequence can be interrupted by one or more non-CTLA4 sequences (*e.g.* a signal sequence, a tag sequence, an epitope, or "the first sequence") without removing B7 affinity, and the resulting interrupted CTLA4 sequence is still "a second sequence" within the meaning of the invention, even though it is non-contiguous.

The second sequence "can bind" to a co-stimulatory ligand of an antigen presenting cell. This
25 binding interaction does not rely on non-specific interactions. Thus the binding will be specific, but this does not imply that the second sequence need bind only the ligand of interest *e.g.* CTLA4 can specifically bind to both B7-1 and B7-2. The binding interaction need not have a very high affinity (*e.g.* the CTLA4/B7-1 interaction has a K_d of $0.42\mu\text{M}$ [42]), but it is specific *e.g.* when compared to irrelevant control proteins.

- 30 Looking beyond co-stimulatory APC/T-cell interactions, the second sequence may be from a T cell receptor (TCR), and particularly from its epitope-binding domain. In this embodiment, the ER-targeted TCR binds and sequesters its MHC peptide epitopes. As a consequence, these epitopes are not presented to the immune system and the immune repertoire can be re-programmed.

Looking beyond T-cell interactions, the second sequence may be from the cell surface receptor CD40
35 found on B-cells, thereby inhibiting cell-surface expression of its ligand (CD40L; CD154), or *vice*

versa (*i.e.* to inhibit cell-surface expression of CD40). More generally, the second sequence can be from any cell surface protein which is the receptor for a protein whose expression is to be inhibited *e.g.* FasL (to block Fas apoptosis).

Further sequences

- 5 In addition to the first and second sequences defined above, a protein of the invention may comprise further sequences (*e.g.* a third sequence, a fourth sequence, *etc.*). For example, the protein may include further sequences to assist in solubility or folding, or to provide binding sites for further proteins, or to help in degradation of the bound ligand, or as a tag to monitor expression (directly or indirectly). Rather than include further sequences to perform such roles, however, they may be
10 provided inherently by the first and/or second sequences.

For example, the invention encompasses the known CTLA4-Ig molecule to which a KDEL motif has been fused. Thus the protein includes a first sequence (ER-retention sequence), a second sequence (extracellular B7-binding portion of CTLA4) and a third sequence (Ig-C γ 1 chain).

- The third sequence may also be a further second sequence. By including more than one second
15 sequence, a protein of the invention can bind to more than one co-stimulatory ligand of an antigen presenting cell *e.g.* the protein could include a CTLA4 sequence for binding B7 and an ICOS sequence for binding B7-H2.

Nucleic acids

The invention also provides nucleic acid which encodes a protein of the invention.

- 20 Nucleic acids of the invention can take various forms *e.g.* single-stranded or double-stranded; circular, branched, or linear; labelled or un-labelled; DNA, RNA, or hybrids thereof; DNA or RNA analogs, such as those containing modified backbones (*e.g.* peptide nucleic acids (PNAs) or phosphorothioates) or modified bases. Thus the nucleic acid could be: a gene or gene fragment, mRNA, tRNA, rRNA, cDNA, recombinant nucleic acids, branched nucleic acids, plasmids, vectors,
25 DNA from any source, RNA from any source, probes, and primers. Where nucleic acid of the invention takes the form of RNA, it may have a 5' cap.

- Nucleic acids of the invention may be part of a vector *i.e.* part of a nucleic acid construct designed for transduction/transfection of one or more cell types. Vectors may be, for example, "cloning vectors" which are designed for isolation, propagation and replication of inserted nucleotides,
30 "expression vectors" which are designed for expression of a nucleotide sequence in a host cell, "viral vectors" which is designed to result in the production of a recombinant virus or virus-like particle, or "shuttle vectors", which comprise the attributes of more than one type of vector. Vectors for the creation of transgenic organisms are preferred.

The nucleic acid may be replicating or non-replicating. The nucleic acid may be integrating or non-integrating. The nucleic acid may be an autonomously replicating episomal or extrachromosomal vector, such as a plasmid.

5 Nucleic acids and vectors of the invention will generally comprise: (i) a promoter; and (ii) a sequence operably linked to the promoter, which encodes a protein of the invention. They may also include one or more of: (iii) a selectable marker; (iv) an origin of replication; and (v) a transcription terminator downstream of and operably linked to (ii).

10 The promoter may be a constitutive promoter or it may be a regulated or inducible promoter. The promoter may be naturally-occurring, but will generally be a chimeric regulatable system incorporating various prokaryotic and/or eukaryotic elements. Various promoter modules can be used to allow various levels of control. For optimising the ability of a transfected APC to induce anergy in a target T cell, in some circumstances it is desirable to allow activation of the T cell to begin, and then block signal 2, rather than blocking signal 2 from the start. Inducible promoters can be used to achieve this goal.

15 Constitutive promoters useful for directing transcription include those from genes coding for glycolytic enzymes, or from β -actin, and allow persistent up-regulation of expression. Viral promoters may also be used *e.g.* from CMV. Inducible and regulated promoters [*e.g.* ref. 43] can allow spatial and/or temporal control of transcription, above and beyond any spatial control achieved by targeted delivery of a vector. Tissue-specific or cell-type-specific promoters facilitate spatial control. Advantageously, the invention uses a promoter which is active in antigen presenting cells, 20 and particularly one which is active in dendritic cells *e.g.* a CD80- or CD86-derived promoter, a CD83-derived promoter [44], a dectin-2 promoter [45], *etc.*

Drug-inducible promoters facilitate temporal control *e.g.* by including cAMP response element enhancers in a promoter, cAMP modulating drugs can be used [46]. Other common regulated 25 systems are based on tetracycline, RU486, ecdysone, rapamycin, *etc.* [43]. In contrast, repressor elements can be included in a vector to prevent transcription in a drug's presence [47]. Spatial and temporal control of gene expression can also be achieved by using a promoter which responds to ionising radiation [*e.g.* refs. 48 & 49].

30 As mentioned above, these various promoters can be used alone or in combination to allow multiple control mechanisms. In addition to the promoter, the nucleic acid may include transcriptional regulatory sequences (*e.g.* enhancers, upstream and/or downstream) to interact functionally with the promoter. Nucleic acid can also include a eukaryotic transcriptional terminator sequence downstream of the coding sequence.

35 Other features of nucleic acids of the invention include: a signal which directs polyadenylation of coding RNAs (*e.g.* from SV40); a selectable marker; an origin of replication; a multiple cloning site;

and an IRES. For non-replicating nucleic acids, the origin of replication is preferably active in prokaryotes but not eukaryotes, thereby facilitating production in convenient prokaryotic systems.

Nucleic acids of the invention can be used, for example: to produce polypeptides; as hybridization probes for the detection of nucleic acid in biological samples; or to generate additional copies of the nucleic acids.

Where a nucleic acid is said to "encode" a polypeptide, it is not necessarily implied that the polynucleotide is translated, but it will include a series of codons which encode the amino acids of the polypeptide.

Cellular materials

The invention also provides a cell containing a protein of the invention. The protein is preferably located in the endoplasmic reticulum lumen. The protein may be complexed to a co-stimulatory ligand.

The invention also provides a cell comprising nucleic acid of the invention. The nucleic acid may be integrated into the chromosome of the cell, or it may be maintained episomally. Techniques for introducing nucleic acids into cells are well known in the art.

The cell is preferably an animal cell *e.g.* a cell from a cow, a sheep, a pig, a horse, or a human.

The cell is preferably an antigen presenting cell. It may be a dendritic cell. Dendritic cells can conveniently be prepared by purification or, advantageously, by differentiation from monocytes.

Preferred cells and tissues are APCs such as dendritic cells and B cells.

Cells which express a protein of the invention may advantageously have further characteristics to assist in down-regulating undesired T-cell responses. For example, APCs may be engineered to express: (a) other MHC molecules to tolerise to the indirect pathway of allorecognition; (b) down-regulatory cytokines to help anergise the T-cells; (c) cell-surface antibodies against molecules on the T-cell surface (*e.g.* anti-CTLA4 scFv on the cell surface may further tolerise T-cells); and/or (d) cytokines to modulate immune responses.

The invention also provides a tissue comprising a cell of the invention.

The invention also provides an organ comprising a cell or tissue of the invention.

The invention also provides an animal (*e.g.* a transgenic animal) comprising a cell, a tissue or an organ of the invention.

30 *Therapy*

The invention provides a method of treating a patient, comprising the step of delivering nucleic acid, protein or cells of the invention to the patient. The invention also provides the use of nucleic acid,

protein or cells of the invention in the manufacture of a medicament. The invention also provides nucleic acid, protein or cells of the invention for use in medicine.

The invention also provides a medicament comprising nucleic acid, protein or cells of the invention in combination with a pharmaceutically acceptable carrier. The term “pharmaceutically acceptable carrier” refers to any pharmaceutical carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition, and which can be administered without undue toxicity. Suitable carriers can be large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers, *etc.* Such carriers are well known to those of ordinary skill in the art. Pharmaceutically acceptable carriers in therapeutic compositions can include liquids such as water, saline, glycerol and ethanol. Auxiliary substances, such as wetting or emulsifying agents, pH buffering substances, and the like, can also be present in such vehicles. Typically, the therapeutic compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid vehicles prior to injection can also be prepared. Pharmaceutically acceptable salts can also be present in the pharmaceutical composition, *e.g.* mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. A thorough discussion of pharmaceutically acceptable excipients is available in reference 50.

Preferred medicaments are aqueous, buffered at pH 7.0±0.5, pyrogen-free, sterile and isotonic with respect to humans.

The invention is particularly suitable for treating autoimmune disease, hypersensitivity reactions and transplant rejection *e.g.* diseases such as lupus, psoriasis vulgaris, asthma, rheumatoid arthritis, multiple sclerosis and allergies. Preferred transplant tissues are those of the eye. More generally, medicaments of the invention can be used for immunosuppression (*e.g.* inducing T cell anergy), immunomodulation (*e.g.* shifting Th1 vs. Th2 responses), and/or immunostimulation, depending on the ligand being targeted by the second sequence. The invention is particularly suitable for inducing tolerance against an antigen(s) of interest. Anergic T cells can regulate immunity, resulting in “linked suppression” [51,52], and so the invention is also suitable for producing regulatory T cells *e.g.* to regulate immunity against tissues which express an antigen of interest. By changing the cell-surface expression of proteins, the invention can also be used to alter the movement, trafficking and recruitment of cells *e.g.* it can inhibit the recruitment of inflammatory cells.

The invention may involve administration of transfected APCs to a patient, or nucleic acid transfer *in vivo* or *in vitro* to APCs followed by administration of the transfected APCs.

Prior to administration, cells may have been loaded with an antigen against which it is desired to induce tolerance. As an alternative, the cells may have been engineered to express such an antigen.

The invention may involve the transplant of cells from one species to cells of another species *i.e.* xenografting or xenotransplantation.

Delivery of nucleic acid

For delivery to a patient, nucleic acid of the invention may be administered as “naked” nucleic acid. More typically, however, it will be packaged for delivery within a vector suitable for use in gene therapy [53].

- 5 The invention can utilise viral vectors [54, 55] (*e.g.* adenovirus vectors [56], adeno-associated virus vectors [57], lentivirus vectors [58], parvovirus vectors, herpesvirus-based vectors, other retroviral vectors, alphavirus vectors, *etc.*), but non-viral vectors [59, 60, 61] are preferred for reduced immunogenicity [62,63]. Suitable delivery systems for vectors include liposomes (*e.g.* cationic or anionic [64 to 68]), microparticles, nanoparticles, condensing agents, bolosomes, lipoplexes, virosomes, cationic polymers, *etc.* Nucleic acids of the invention may therefore include suitable packaging signals.

Viral and non-viral vectors can be administered to a patient in various ways *e.g.* by hand-held gene transfer particle gun [69], or by injection of the vector.

Delivery of the vector may be systemic, but will generally be targeted to antigen presenting cells.

- 15 Targeting may be receptor-mediated (*e.g.* using immunoliposomes), as described in, for example, references 70 to 75. As an alternative, targeting may involve direct delivery to target tissue. Another targeted delivery method (particularly for non-viral vectors) involves administering the vector to the body and “activating” it specifically in APCs. Immunoliposomes targeted to monocytes by anti-CD71 monoclonal antibody is a preferred method of targeting.

- 20 Therapeutic compositions containing a nucleic acid are typically administered in a range of about 100 ng to about 200 mg of DNA for local administration in a gene therapy protocol. Concentration ranges of about 500 ng to about 50 mg, about 1 µg to about 2 mg, about 5 µg to about 500 µg, and about 20 µg to about 100 µg of DNA can also be used during a gene therapy protocol. Factors such as method of action and efficacy of transformation and expression are considerations which will affect the dosage required for ultimate efficacy. Where greater expression is desired over a larger area of tissue, larger amounts of vector or the same amounts re-administered in a successive protocol of administrations, or several administrations to different adjacent or close tissue portions may be required to effect a positive therapeutic outcome. In all cases, routine experimentation in clinical trials will determine specific ranges for optimal therapeutic effect.

- 30 Delivery of nucleic acid can occur *in vivo* or *ex vivo*. *Ex vivo* gene therapy requires the isolation and purification of patient cells, the introduction of nucleic acid of the invention (*e.g.* by electroporation, or any other suitable *in vitro* transfection method) and introduction of the genetically altered cells back into the patient. In contrast, *in vivo* gene therapy does not require isolation and purification of a patient’s cells.

Combination therapy

The invention may be used in conjunction with further immunosuppressive techniques. In a transplantation situation, for instance, the invention could be used together with one of the approaches described in references 7, 76 and 77, or could be used in conjunction with conventional immunosuppressive drugs (*e.g.* CyA, *etc.*).

The invention may be used in conjunction with up-regulatory or down-regulatory cytokines to modulate T-cell responses, or with immunoglobulins which recognise T-cell surface proteins. Such co-administered molecules can further assist *e.g.* in anergy induction.

Receptor/ligand investigations

The invention also allows the cell-surface proteins to be identified and/or investigated. Taking the CTLA4 protein as an example, and imagining that its function is unknown, the skilled person could express CTLA4 according to the invention in various cell lines and study the phenotype of the cells. Reduced CD80 expression would be noted, which would identify CD80 as a binding partner for CTLA4. The two proteins can then both be investigated to study phenotypic changes and their individual and joint functions can be inferred. This method is advantageous when compared to siRNA methods or intrabodies because it requires no *a priori* knowledge of the protein whose expression is being blocked (*e.g.* CD80).

The invention therefore provides a method for investigating a cell-surface protein, comprising the steps of: (a) expressing a polypeptide in one or more cell types, wherein the polypeptide comprises (i) at least the extracellular binding domain of the cell-surface protein, and (ii) a sequence which causes the polypeptide to be retained in the endoplasmic reticulum; and (b) comparing the phenotype(s) of the one or more cell types with control cells which do not express the polypeptide.

Dendritic cell differentiation

It has been found that monocytes can be transfected with greater efficiency than dendritic cells. The invention therefore provides a method for preparing a genetically-modified dendritic cell, comprising the steps of: (a) genetically modifying a monocyte; and (b) differentiating the monocyte into a dendritic cell. Methods for differentiating monocytes into dendritic cells in step (b) are known in the art, and include: culture in the presence of GM-CSF and IL-4 [78-80], optionally also with TNF- α [81] or IFN- γ [82,83]; culture in the presence of GM-CSF and IL-13 [84]; culture (particularly serum-free culture) in the presence of bacterial LPS, TNF- α or calcium ionophore [85]; culture with GM-CSF and bacterial LPS [86]; culture with IL-3 and IFN- β [87]; culture with cutaneous fibroblasts [88]; see more generally in reference 89.

Step (a) will typically involve the use of a nucleic acid vector, as described above. Receptor-mediated transfection of monocytes may be based on their cell-surface CD71 receptors.

Transfected dendritic cells have been described for various purposes *e.g.* cancer vaccination [90] and other types of vaccination. The invention allows these dendritic cells to be prepared more efficiently.

General

When referring to co-stimulatory interactions between APCs and T-cells, the “ligand” is the protein on the APC surface and the “receptor” is the protein on the T-cell surface. More generally, however, the designation of “ligand” and “receptor” in interactions between cell-surface proteins is essentially arbitrary, and so the terms can be used interchangeably *e.g.* the invention can target CTLA4 to the ER and thus prevent cell-surface expression of CD80, or it can target CD80 to the ER and thus prevent cell-surface expression of CTLA4.

The term “comprising” means “including” as well as “consisting” *e.g.* a composition “comprising” X may consist exclusively of X or may include something additional *e.g.* X + Y.

10 The term “about” in relation to a numerical value x means, for example, $x \pm 10\%$.

The word “substantially” does not exclude “completely” *e.g.* a composition which is “substantially free” from Y may be completely free from Y. Where necessary, the word “substantially” may be omitted from the definition of the invention.

The term “antibody” includes any suitable natural or artificial immunoglobulin or derivative thereof.

15 In general, the antibody will comprise a Fv region which possesses specific antigen-binding activity. This includes, but is not limited to: whole immunoglobulins, antigen-binding immunoglobulin fragments (*e.g.* Fv, Fab, F(ab')₂, *etc.*), single-chain antibodies (*e.g.* scFv, or antibodies from camelids and nurse sharks), oligobodies, minibodies, diabodies, chimeric antibodies, humanized antibodies, veneered antibodies, camelised antibodies, phage-displayed antibodies, *etc.*

20 Proteins and nucleic acids of the invention are preferably provided in isolated or substantially isolated form *i.e.* substantially free from other proteins or nucleic acids (*e.g.* free from naturally-occurring proteins or nucleic acids), generally being at least about 50% pure (by weight), and usually at least about 90% pure or more.

BRIEF DESCRIPTION OF DRAWINGS

25 Figure 1 shows FACS data from M1 cells. Figure 2 shows the results of (2A) a MLR assay and (2B) a peptide-specific T cell response assay using the same cells.

Figure 3 shows FACS data from HLA-DR1-expressing B cells. Expression of CD40, CD54, MHC II CD80, CD86, IgG and CD19 is shown. Figure 4 shows anti-c-myc western blotting, confirming CTLA4-KDEL expression in these cells. Figure 5 shows the results of a MLR assay using these cells.

30 Figure 6 shows the results of a peptide-specific T cell response assay using the same cells. Figure 7 shows the levels of IL-4, IFN γ and IL-10 in the same cells, measured by ELISA.

Figure 8 shows FACS data from immature and mature dendritic cells. MHC-I, MHC-II, CD80, CD86, CD83, CD54, CD40, CD11c and CD14 are shown. The ability of these cells to stimulate an allogeneic MLR by naïve T cells is shown in Figure 9, and the levels of IL-4, IFN- γ and IL-10 are shown in Figure 10.

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Figure 11 shows ^3H -thymidine incorporation by T cells. The initial response is shown in 11A. A re-challenge after 6 days of rest is shown in 11B. The anergy shown in 11B can be reversed by addition of rIL2, as shown in 11C.

Figure 12 shows ^3H -thymidine incorporation ($\text{cpm} \times 10^3$) by naïve T cells after co-culture with mature dendritic cells. The dendritic cells were either from the same donor (12A & 12C) or from a different donor (12B & 12D). Figures 12C & 12D show results in the presence of rIL2.

Figures 13 & 14 show the effect of an IDO antagonist on T cell proliferation.

Figure 15 shows RT-PCR analysis of IDO and β -actin in dendritic cells.

Figure 16 shows T cell proliferation of different subsets of T cells.

Figure 17 shows construction of the CTLA4-KDEL construct.

Figure 18 shows division of T cells labelled with CFSE. Arrows show cell divisions.

Figure 19 shows ^3H -thymidine incorporation during T cell proliferation. Cells were either untransfected (black), mock-transfected (white) or CTLA4-KDEL transfected (grey).

Figure 20 shows ^3H -thymidine incorporation during T cell proliferation ($\text{cpm} \times 10^3$), illustrating the regulatory phenotype of CTLA4-KDEL transfected T cells. Figure 21 shows cytokine production by the T cells, again showing their regulatory phenotype. Figure 22 shows the effect of transwell separation on the regulatory phenotype.

Figure 23 shows marker expression on endothelial cells expressing CTLA4-KDEL. Figure 24 shows stimulation of allogeneic MLR by transfected endothelial cells, and figure 25 shows the cytokine responses induced by the endothelial cells.

Figure 26 shows expression of CD80/86 by Western blotting. Figure 27 shows cell lysates immunoprecipitated with anti-CTLA4 (left panel) and anti-c myc (right panel) prior to western blotting and probing with anti-CD80, CD86 or CTLA4 mAb. Figure 28 shows mRNA levels for a range of heat shock proteins, as assessed by RT-PCR. Figure 29 shows Western blots of cell lysates that were probed with antibodies against phosphorylated PERK and phosphorylated eIF-2 α proteins.

Figure 30 shows expression of phosphorylated retinoblastoma protein (ppRb), Cyclin E, Cyclin D2, Cdk4 and p27^{kip1} proteins as analysed by western blotting.

Figure 31 shows levels of p27^{kip1} as analysed by Western blotting. The numbers under the blot refer to densitometric analysis of the bands.

Figure 32 shows expression of cell surface CD80/86 and I-A^d in murine DCs were transfected with CTLA4-KDEL or mock KDEL constructs compared by flow cytometry. The FACS analysis was performed on different days post-transfection, as indicated on the left of the figure. Figure 33 shows Western blotting of CTLA4-KDEL-transfected murine DCs for PERK and its downstream substrate eIF-2 α . Figure 34 shows the levels of T cell division in T cell receptor transgenic T cells that had been given to an animal before being given antigen pulsed untransfected, mock KDEL-transfected DCs, CTLA4-KDEL-transfected DCs and no DCs. Figure 35 shows T cell proliferation in response

to antigen of spleen cells from animals treated with CTLA4-KDEL-transfected DCs compared with cells from animals treated with untransfected DCs, mock-KDEL-transfected DCs and those that received T cells alone.

Figure 36 shows expression of ICOS L and PD-L1 on human DC transfected with CTLA4-KDEL.

5 MODES FOR CARRYING OUT THE INVENTION

Construction of CTLA4-KDEL

The human CTLA4 gene from pcDNA3-CTLA4 plasmid was amplified by PCR with the primers SEQ ID NO^s: 16 & 17 (full-length) or SEQ ID NO^s: 16 & 18 (extracellular portion), with *Bss*HII and *Not*I restriction sequences at 5' end and 3' end respectively. The gene was then cloned into
10 pCMV/*myc*/ER (Invitrogen) into *Bss*HII and *Not*I sites using standard protocols. The sequence of the final plasmid is SEQ ID NO: 20 (*Bss*HII site:784-789; *Not*I site: 1464-1471), and the encoded CTLA4-KDEL protein has amino acid sequence SEQ ID NO: 19. The N-terminal ER signal peptide upstream of the CTLA4 sequence and the C-terminal sequences downstream of the CTLA4 sequence are derived from the vector. The overall strategy is shown in Figure 17. A further CTLA4-KDEL
15 gene was constructed which encodes a protein without the CTLA4 C-terminus tail (SEQ ID NO: 21). This latter construct was found to give improved B7 knockout, as shown below.

Transient CTLA4-KDEL transfection of artificial APCs.

The CTLA4-KDEL construct was transiently transfected into M1, human fibroblast cells transfected with HLA-DR1 (DRA and DRB1*0101 genes), B7-1 and/or B7-2 genes. The modified fibroblast
20 cells act as antigen presenting cells.

M1 cells already transfected with DR1 and B7.1 were transiently transfected with mock-KDEL (pCMV-ER, containing empty KDEL) and CTLA4-KDEL constructs or with a plasmid encoding EGFP. The cells were cultured and maintained as described in references 91 & 92. CD80 or EGFP expression was determined by flow cytometry. As shown in Figure 1, cells transfected with
25 CTLA4-KDEL showed reduced expression of B7-1 (CD80).

These transfectants were tested for their ability to stimulate primary allogeneic MLR (mixed lymphocyte reaction). Allogeneic T-cells were isolated and purified using protocols previously described [93]. The purified cells were resuspended in medium ready for proliferation assay and accessory cell contamination was assessed by culture with 1 µg/ml PHA in a 48 hour assay. The
30 purified cells (1x10⁵/well) were cultured in the presence of 180-Gy x-irradiated B-LCL or mitomycin-C treated M1 transfectants and irradiated DCs in a total volume of 200 µl. Cultures were pulsed with 1 µCi/well of ³H-thymidine for 16 hours. As expected, the transfectants lacking B7-1 and B7-2 failed to stimulate the allogeneic cells (Figure 2A), and cells expressing B7-1 and/or B7-2 transfected with CTLA4-KDEL showed a reduced ability to stimulate the T-cells when compared to
35 untransfected cells or those transduced with the mock-KDEL control plasmid.

The transfectants were also tested for their ability to stimulate a T-cell clone specific for HA peptide. The HC3 T-cell clone [94,95], specific for HA 100-105 restricted by DRB1*0101, was used to test peptide specific response using the HA peptide at various concentrations with ^3H -thymidine incorporation being assessed after 3 days. T-cells were purified by isolation on Lymphoprep™ (Axis-Shield PoC AS, Oslo, Norway) gradient 7 days after re-stimulation and washed five times by low speed centrifugation ($210 \times g$, 5 min) before use, to exclude contamination by accessory cells. T cells from the clone (10^4 cell/well) were cultured in the presence of 180 Gy x-irradiated B-LCL (3×10^4 /well) or mitomycin-C treated M1 transfectants (3×10^4 /well) in flat well microtiter plates, in a total volume of 200 μl . The stimulator cells were pre-pulsed overnight with peptide and then washed to remove any soluble peptide. Wells were pulsed with 1 μCi of ^3H -thymidine after 48 h. Transfectants lacking B7-1 and B7-2 failed to stimulate the peptide-specific T-cells (Figure 2B), and cells expressing B7-1 and/or B7-2 transfected with CTLA4-KDEL showed a reduced ability to stimulate the T-cells when compared to untransfected cells or those transduced with the mock-KDEL control plasmid.

Therefore the artificial APCs which expressed CTLA4-KDEL showed reduced B7 activity.

Stable CTLA4-KDEL transfection of B cells.

Having established the effect of CTLA4-KDEL construct in a transient expression system, its effect in stably transfected B cell lines (HLA-DR1 and DR11, homozygous) was determined.

EBV-transformed B-lymphoblastoid cell lines (B-LCL) from the 10th International Histocompatibility Workshop, were cultured and maintained as previously described [94,95]. The cells were stably transfected by standard electroporation methods [96] with CTLA4-KDEL or mock-KDEL, and expression of CD40, CD52, MHC-II, CD80, CD86, IgG & CD19 was analysed by flow cytometry. For each transfection experiment, the liposomes and Tfx-50™ reagents were prepared in accordance to manufacturer's instructions (Promega). The transfection complex was made by addition of the plasmid DNA to opti-MEN medium (Invitrogen) followed by the Tfx-50™ and heat-treated anti-CD71 mAb, OKT9 as previously described [99,97]. The optimal OKT9 concentration was 60 $\mu\text{g}/\text{ml}$. The optimal ratio of DNA to Tfx-50™ reagents was determined to be 1:3 (w:v).

Transfection with CTLA4-KDEL did not affect the expression of molecules such as MHC class II, CD40, CD54, CD19 and IgG. However, the CD80 and CD86 expression was completely abolished in B cells expressing HLA-DR1 (Figure 3). Similar results were seen on B cells expressing HLA-DR11.

To confirm expression of CTLA4-KDEL, the c-myc protein tag incorporated in the construct was used for western blotting. Cell lysates were prepared and loaded onto 12% SDS-PAGE gel before electroblotting onto a nitrocellulose membrane. Non-specific binding was blocked using 5% powdered milk in PBS-Tween. The anti-myc Ab, 9E10 (10 $\mu\text{g}/\text{ml}$) or anti- β -actin was applied in blocking buffer and incubated for over an hour. HRP-conjugated rabbit anti-mouse Ab was then applied in a blocking buffer at 1:2000 dilution and incubated at room temperature for 30 min before the blot was developed using enhanced chemiluminescence. 54 kDa products were detected in the

stably transfected B-cells (Figure 4) but not in the supernatant. Immunocytochemical staining confirmed the expression of MHC-II, CD19, CD40, CD80, CD80 and BCR (IgG). All cells contained β -actin.

The B-cells were tested in a MLR assay. B-cell lines expressing HLA-DR1 or DR11 were used as stimulators in MLR assay, having been transfected with the mock-KDEL or the CTLA4-KDEL plasmid, or with no transfection. As a positive control, 10 μ g/ml CTLA4-Ig was added to allogeneic MLR. ^{3} H-thymidine incorporation was determined after 5 days. The stably transfected B-cell lines, showed a reduced ability to stimulate a MLR (Figure 5), and the inhibition was comparable than that seen following addition of soluble CTLA4-Ig.

Peptide-specific responses of the B-cells were tested using various T cell clones (HC3, NF4, MJ34 and MJ60). The T cell clones were co-cultured with HLA-DR1- and HLA-DR11- expressing B cells which had previously been incubated with different concentrations of HA peptides. ^{3} H-thymidine incorporation was determined after 3 days. The stably transfected B-cell lines were shown to be unable to stimulate proliferation of four T-cell clones (HC3, NF4, MJ60 & MJ36), specific for HA peptides in the context of either HLA-DR1 or DR11 (Figure 6).

The supernatants from the HC3 clone and allogeneic T-cells co-cultured with either untransfected HLA-DR1 expressing B cells (U) or stably transfected with CTLA4-KDEL (C) and mock-KDEL (M) were collected on day 4. The levels of IL-4, IFN- γ and IL-10 were measured using ELISA [98]. The T-cells stimulated with CTLA4-KDEL exposing to B-cells showed a different pattern of cytokine expression to those stimulated with unmodified and control B-cells with increased levels of IL4 and IL-10 and reduced IFN- γ , typical of Th2 skewing (Figure 7). A similar pattern is found in both primary allospecific response and in the HC3 T-cell clone.

CTLA4-KDEL transfection of dendritic cells

Dendritic cells were transfected with CTLA4-KDEL with non-viral vectors, using anti-CD71 immunoliposomes [99,100]. Transfection efficiency was low (~10%) for DCs, but was higher (20-30%) for monocytes (20-30%). Transfection was thus performed for monocytes, which were then differentiated into DCs for further study [101].

Monocytes were transfected with either CTLA4-KDEL or mock-KDEL, or were left untransfected. Monocytes were then differentiated into DC with GM-CSF and IL4. Antibiotic G418 was added to transfected cells on day 2, which allowed selection of transfected DCs. On day 8, the cells were either matured with 20 ng/ml TNF- α , 20 ng/ml IL-1 β , 20 ng/ml LPS, 10 ng/ml PGE2, 20 ng/ml IFN- γ for 48 hours, or they were maintained in immature form. At 10 days, the expression of MHC-I, MHC-II, CD80, CD86, CD83, CD54, CD40, CD11c and CD14 was analysed using flow cytometry.

The expression of MHC class I, Class II, CD80, CD83 and CD86 (Figure 8 — i, iii, v & vii) and CD11c, CD14, CD40 and CD54 expression (Figure 8 — ii, iv, vi & viii) was identical in DCs that had been transfected with mock-KDEL or untransfected (not drug selected) indicating that the

transfection and selection regimen had not altered the phenotype of cells. This was seen in both immature DCs (iDC) and DC that had been matured with a cocktail of pro-inflammatory cytokines (mDC). However, DCs (both immature and mature) that had been transfected with CTLA4-KDEL showed no expression of CD80 and CD86. FACS data were confirmed by immunochemical staining.

- 5 RT-PCR showed that the mRNA levels of CD80/86 and other molecules were unaffected by CTLA4-KDEL expression (data not shown), while western blotting (Figure 26) and immunocytochemistry (data not shown) showed little expression of CD80/86, suggesting rapid degradation. In order to identify the likely degradation pathway, we treated CTLA4-KDEL-transfected DCs with proteasome inhibitors, which resulted in increased levels of CD80/86, indicating that degradation is a
- 10 proteasome-dependent process (Figure 26). In order to demonstrate co-localisation of CTLA4-KDEL and CD80/86, the cell lysates were immunoprecipitated with anti-CTLA4 (left panel Figure 27) and anti-*c myc* (right panel Figure 27) prior to western blotting and probing with anti-CD80, CD86 or CTLA4 mAb. This showed that CTLA4-KDEL was colocalized with CD80 and CD86 proteins, as was also suggested by deconvolution microscopy (data not shown).
- 15 One potential problem with the use of KDEL-tagged fusion proteins to form complexes in the ER is that this might stress the cell, leading to non-specific effects. We therefore determined whether expression of CTLA4-KDEL and the formation of the resulting complex in the ER induced a detectable response. No marked increase in mRNA levels was seen for a range of heat shock proteins over that seen with cells transfected with GFP, as assessed by RT-PCR (Figure 28).
- 20 More importantly there was no upregulation of phosphorylation of the ER stress responsive enzyme PERK, or its downstream molecule eIF-2 α [102]. This was shown using Western blots that were probed with antibodies against phosphorylated PERK and phosphorylated eIF-2 α proteins. These respond to stress in the endoplasmic reticulum with a number of downstream effects, such as inhibition of protein synthesis. They are thought to be a response element for viral infection. As a
- 25 control in these experiments, DCs were also transfected with GFP or with an intrabody (anti-VCAM-1 scFv fused to KDEL [103]) directed against VCAM-1; both GFP and the intrabody induced ER stress (Figure 29).

In the case of human DCs, we were therefore unable to demonstrate any significant upregulation of heat shock proteins. Nor was any upregulation of PERK (the ER stress response protein) and eIF-2 α

30 observed. It thus appears as if the human dendritic cells are not affected adversely by expression of CTLA4-KDEL. This was in contrast to cells transfected with intrabody (scFv-KDEL fusion protein [103]), which showed upregulation of PERK and eIF-2 α , possibly due to poor folding of the intrabody in the ER, with the subsequent activation of the ER stress pathway. The use of a surface ligand, which is normally expressed in the ER, may be an advantage in this respect.

- 35 The ability of these DCs to support stimulated mixed lymphocyte response by allogeneic T cells was tested. The mDC and iDCs expressing CTLA4-KDEL failed to support the proliferation of T cells

(Figure 9). This failure to support proliferation was also seen in FACS analysis (Figure 18) and by ^3H -thymidine incorporation (Figure 19). The only cells not to proliferate are $\text{CD}25^{+\text{ve}}$ cells, which are regulatory and seldom divide.

The supernatants from allogeneic T cells co-cultured with either untransfected DCs (U) transfected with CTLA4-KDEL (C) and mock-KDEL (M) were collected on day 4. The levels of IL-4, IFN- γ and IL-10 were measured using ELISA. DCs produced increased IL-4 and decreased IFN- γ when compared with those exposed to control DCs (Figure 10). For T-cells stimulated with B cells expressing CTLA4-KDEL, however, no IL-10 production was seen.

T-cell anergy

The transfected B cells and DCs were tested to see if the hyporesponsive T cells generated above had been rendered anergic.

10^6 cells of HC3 clone were co-cultured with an equal number of HLA-DR1- expressing B-cells, which were either non-transfected, CTLA4-KDEL and mock-KDEL transfected, in the presence of HA peptide. After 3 days, the proliferation of the clones was determined by ^3H -thymidine incorporation. The clone was hyporesponsive during their first encounter with the HA antigen presented by CTLA4-KDEL transfected B cells (Figure 11A).

After 2 days the clone was isolated and the expression of phosphorylated retinoblastoma protein (ppRb), Cyclin E, Cyclin D2, Cdk4 and $\text{p}27^{\text{kip1}}$ proteins analysed by western blotting (Figure 30). In addition the co-association of cdk2, Cyclin D2, cdk4 with other proteins was assessed by immunoprecipitation with appropriate Ab (IP Ab) followed by western blotting and probing with anti Cyclin E or Cyclin D2 Ab (blot Ab) (Figure 30). The enzymatic activity of the proteins was determined using *in vitro* kinase reactions, with Histone H1 or a retinoblastoma-GST (Rb-GST) fusion protein. The lack of expression of phosphorylated retinoblastoma protein and Cyclin E, as well as lack of functional kinase activity by Cyclin D2, cdk2 and cdk4, as well as reduced association of cdk2 and cdk4 with Cyclins E and D2, respectively, demonstrates that T cells exposed to CTLA4-KDEL-transfected B cells in the presence of HA peptide were arrested at the early G1 phase of the cell cycle. The cells also expressed a high level of $\text{p}27^{\text{kip1}}$ typical of anergic cells.

The T-cells were then rested for 6 more days, followed by a re-challenged culture with an equal number of unmodified HLA-DR1- expressing B cells and HA peptide. As can be seen in Figure 11, T-cells were hyporesponsive during first culture with B-cells expressing CTLA4-KDEL. Upon re-challenge with B cells expressing co-stimulatory molecules, they maintained unresponsive (Figure 11B) indicating that they had become anergic. As expected from previous studies [104], this anergy could be reversed by addition of 10U/ml rIL2 (Figure 11C).

In further experiments, a bulk culture of T cells (10^7) was co-cultured with DCs (10:1 ratio) which were not transfected or transfected either with CTLA4-KDEL or mock-KDEL. After 5 days proliferation of T-cells was measured. The T-cells were then maintained and cultured for 5 days. On

the 10th day, the clones were re-challenged with either mDCs from the same donor (Figure 12A) or from a third party (Figure 12B) at 5:1 ratio in the presence or absence of 10 U/ml rIL2. Proliferation of T cells was determined by ³H-thymidine incorporation on day 3, day 5 and day 7 in order to differentiate between a primary and secondary response. As can be seen, T-cells exposed to CTLA4-KDEL failed to respond to mDCs from the same donor, though they did respond to third party DCs with a kinetic typical of a primary response. T-cells stimulated with control DCs in the first culture showed a rapid response to DCs from the same donor typical of a secondary immune response. The anergised T cells had high levels of p27^{kip1} as analysed by Western blotting (Figure 31) and the majority of T cells were arrested at the early G1 phase of the cell cycle (data not shown), consistent with their anergic phenotype [105, 106]. As expected the hyporesponsiveness of the T-cells could be reversed by addition of exogenous rIL-2 (Figures 12C & 12D).

Induction of regulatory T cells

Having established that T cells exposed to CTLA4-KDEL-transfected DCs were anergic, the same cells were tested for their *in vitro* immunoregulatory ability. The irradiated anergic cells were added to a culture of the T cells (from the same donor as anergic cells) and a fully-competent allogeneic DC (from the same donor as DC of the primary culture when anergic cells were generated) or third party DC (complete DR-mismatched) at a ratio of 1:1, 1:2.5 and 1:5. The supernatants showed a skew toward Th2 profiles of cytokine production with high levels of IL-10 production.

Results are shown in Figures 20 & 21. T cells that had been incubated with the various DCs shown on the x axis of Figure 20 were added back into a new MLR with competent DCs and fresh T cells. The added cells suppressed the response of fresh allogeneic T cells, but not the of fresh third party T cells *i.e.* the effect is specific. The cytokine production shown in Figure 21 confirms the data.

To investigate the mechanism of this regulation, transwell experiments were used [107, 108] to separate anergic T cells from allogeneic MLR. The anergic T cells mediated their regulatory function in a manner which was partially dependent on cell-cell contact, as the transwell separation partially reversed their regulatory functions, whereas the addition of anti-IL-4, IL-10, TGFβ neutralising mAbs or anti-CTLA4 mAb did not have any effect on their regulatory function (Figure 22). Regulation is thus mediated by cell contact rather than by cytokine release.

The fact that T cells that have been incubated with CTLA4-KDEL transfected DC, when added to a fresh mixed lymphocyte response, inhibited the proliferation of these T cells (but not third party T cells) means that they can be considered regulatory cells. We have spent some time characterising the nature of the regulation. The importance of this result is that it means that the cells can mediate linked suppression. In other words, if T cells are tolerised to antigen X then they will suppress T cells tolerant to antigen Y on the same antigen presenting cell. This is clearly important as it means in the context of transplantation it is possible to tolerise to one HLA molecule and then spread that tolerance to others. We can also in autoimmune disease tolerise to a peptide and spread that tolerance

to all the antigens. In addition, it suggests *in vivo* that not all the antigen reactive T cells need to see the modified dendritic cells for the treatment to work.

CTLA4-KDEL transfection of endothelial cells

Using the CTLA4-KDEL construct, a stable endothelial cell (EC) line expressing CTLA4-KDEL was generated using G418 selection. CTLA4-KDEL-transfected ECs were shown to be deficient of CD80 following stimulation with cytokines or CD40 ligation (Figure 23). Expression of other surface markers such as CD54, CD105, CD54 and MHC class II was unaffected by CTLA4-KDEL transfection up to 8 hours after stimulation.

To show the selected cells were endothelial in phenotype, the CTLA4-KDEL-transfected cells were stained intracellularly for Von Willebrand factor and extracellularly for CD31 (Figure 23). Similar observations were obtained for mock-KDEL-transfected and untransfected cells.

The functional consequence of CTLA4-KDEL transfection in activated ECs was tested in completely mismatched allogeneic MLR (Figure 24). Transfection of CTLA4-KDEL resulted in poor stimulation of naïve T cells. T cells stimulated with CTLA4-KDEL-expressing ECs (activated with CD40 ligation) also showed a different pattern of cytokine expression from those stimulated with either unmodified but activated or mock-KDEL ECs (activated with CD40 ligation) with increased levels of IL-4 and IL-10 and reduced IFN- γ secretion, a profile typical of Th2 skewing (Figure 25). This is very similar to profiles seen on B cells. Similar Th2 skewing has been reported for T cells rendered anergic by incubation with non professional antigen presenting cells or T cells, and has been reported *in vivo* following induction of anergy [109-111]. The expression of ICOS L and PD-L1 on DC transfected with CTLA4-KDEL was unaltered (Figure 36), and so this apparent Th2 bias may due to the unopposed (without CD28-CD80/86) costimulation through ICOS-ICOS L [112] and PD-PD-L1 [113] interactions, as has been previously described [112, 113]. This is in contrast what was seen when CTLA4-Ig was added to cultures containing control DC, which showed the expected reduction in IFN γ , IL4 and IL10 (data not shown), which may in DC be associated with upregulation of IDO (not seen in CTLA4-KDEL transfected cells).

Indoleamine 2, 3-dioxygenase (IDO) enzymes

Previous work has shown that soluble CTLA4-Ig can interact with CD80/86 on DC and trigger the indoleamine 2, 3-dioxygenase (IDO) enzymes [114]. This may contribute to T-cell unresponsiveness by breaking down tryptophan that is essential for T-cell replication. To determine the relative importance of this mechanism, proliferation of T cells to DCs transfected with CTLA4-KDEL was compared to that seen with wild-type DCs in the presence of soluble CTLA4-Ig. The former situation represents failure to stimulate T-cell proliferation due to lack of co-stimulation, while in the latter upregulation of IDO may be important.

CTLA4-KDEL or mock-KDEL transfected DCs were co-cultured with allogeneic T-cells in the presence of various concentrations of human Ig or CTLA4-Ig. ^3H -thymidine incorporation was

measured on day 5. The effect of the IDO antagonist, 1-MT, was determined by co-culture of equal number of CTLA4-KDEL or mock-KDEL transfected DCs with allogeneic T-cells in either the presence of 10 µg/ml CTLA4-Ig or 10 µg/ml human Ig and in the presence of MT-1. ³H-thymidine incorporation was determined on day 5.

- 5 As shown in Figure 13, T-cells stimulated with DCs expressing CTLA4-KDEL showed approximately 10% proliferation compared to wild-type DCs. CTLA4-Ig was able to induce the proliferation to wild type cells (but not CTLA4-KDEL expressing cells) still further, indicating that it works by more than simple presence of blocking of CD80/86 interaction. This increased hyporesponsiveness could be restored to that seen in DCs expressing CTLA4-KDEL by addition of
10 500µM 1-MT (Figure 14), indicating that this additional pathway is due to upregulation of IDO. While CTLA4-Ig can operate both blocking and up-regulation of IDO, it is clear that the main effect is due to blocking.

Using a RT-PCR assay [115,116], the upregulation of IDO transcription in DCs following CTLA4-Ig crosslinking was studied. Using a negative bead selection on CD4+ T-cells, DCs were isolated from
15 allogeneic MLRs, which were treated with CTLA4-Ig or human Ig or in allogeneic MLRs set up with unmodified DC (U), mock-KDEL (M) and CTLA4-KDEL (C) were analysed for IDO transcription. As shown in Figure 15, RT-PCR of the DCs isolated from MLRs on day 3, 5 and 7 indicates that there was a marked up-regulation of IDO transcription in DCs from CTLA4-Ig treated MLR but not in human Ig treated MLR or in MLRs set up with unmodified, mock-KDEL or CTLA4-KDEL
20 transfected DCs.

The hyporesponsiveness caused by CTLA4-KDEL transfected DCs is not inhibited by 1-MT, indicating that this strategy is independent of IDO upregulation. Further evidence of this was obtained in separate experiments. In order to ensure that the protocols for DC preparation were not biased toward IDO+ DC, adherent and non-adherent DCs were separated. The non-adherent DCs
25 were 90% CD123+ and CCR6+. CD123+ CCR6+ DCs and CD123- CCR6- DCs were tested with the CTLA4-KDEL and mock-KDEL as previously described [117]. Thus non-adherent CD123, CCR6 positive subset of DCs (>90% purity) which contribute to T-cell hyporesponsiveness by an IDO-dependent mechanism were separated out. These different populations of DC either transfected with CTLA4-KDEL or mock-KDEL were used as stimulators in a MLR assay in the presence or
30 absence of 1-MT. ³H-thymidine incorporation was determined on day 5. As expected, T-cells failed to proliferate to CD123+ CCR6+ DCs except in the presence of 1-MT (Figure 16). However CTLA4-KDEL expressing DCs failed to support proliferation of either DC subset in the presence or absence of 1-MT confirming that they operate in IDO independent fashion.

Effects of CTLA4-KDEL-transfected DC in vivo.

- 35 As a prelude to *in vivo* experiments, murine DCs were transfected with CTLA4-KDEL or mock KDEL constructs and the expression of CD80, CD86 and I-Ad compared by flow cytometry to untransfected cells on day 2 and 3. As expected, there was loss of expression of cell surface CD80/86

(Figure 32). However, we also noted a reduction of expression of H2-A^d (Figure 32) and CD11c (data not shown) 2 days after transfection, though this was transient and expression had largely recovered by 3 days. In contrast to our human data, using Western blotting of samples taken at the same time points, we showed that CTLA4-KDEL-transfection of murine DCs resulted in a transient upregulation of ER-stress related proteins such as PERK and its downstream substrate eIF-2 α (Figure 33). The loss of expression of H2-A^d and CD11c may be a result of the anti-proliferative [118] and anti-protein synthesis [119] consequences of eIF-2 α activation.

To determine the effect of administering CTLA4-KDEL-transfected DCs *in vivo*, we partially reconstituted BALB/c mice with CFSE-labelled T cells from DO11.10 mice transgenic for a chicken ovalbumin (OVA)-specific, H-2A^d restricted TCR (5×10^6 T cells). One day later CTLA4-KDEL transfected or control DCs loaded with OVA-peptide were used to immunise the animals. Three days after DC administration the local lymph nodes were harvested and the proliferation of the OVA specific T cells determined by flow cytometric analysis for CFSE-expression on cells gated for the expression of the TCR-clonotype. As shown in Figure 34, high levels of T cell division were seen in animals given either untransfected or mock-KDEL-transfected DCs, however following immunisation with CTLA4-KDEL-transfected DCs the division was similar to that seen in animals given no DCs. To determine if the T cells from the animals treated with DCs were anergic, splenocytes of these same mice were incubated with OVA-peptide and their proliferation was measured with ³H-thymidine incorporation at 48, 72 and 108 hours. As shown in Figure 35, no proliferation was seen with spleen cells from animals treated with CTLA4-KDEL-transfected DCs at any of the time points tested, while good proliferation was seen with cells from animals treated with untransfected DCs, mock-KDEL-transfected DCs or those that received T cells alone.

This indicates that the animals had been made tolerant to the ovalbumin antigen, and thus that the described system of the present invention works *in vivo*.

Conclusions and discussion

Human CTLA-KDEL in human APCs (artificial APCs, B cells and DCs) is able to block B7 expression of the APCs, and antigen-reactive T-cells (both allospecific and peptide specific) are rendered hyporesponsive to antigen presented by these cells and become anergic.

In this study, we have used a fusion protein between CTLA4 and the ER-retention signal, KDEL [120, 121] to prevent expression of CD80/86 by APCs. This strategy is similar to the use of intrabodies consisting of KDEL fused to antibody fragments to prevent cell surface expression of target molecules [122, 123] or intrakines (chemokine-KDEL fusion), to block expression of chemokine receptors [124, 125]. The advantage of using a cell surface molecule such as CTLA4, rather than antibody is that the construct is capable of blocking expression of all its ligands (in this case CD80 and CD86), rather than needing a separate antibody for each target. In addition, the immunogenicity of the construct would be low, as the protein is derived from human sequences. This is particularly important as we have shown that degradation of CD80/86 in CTLA4-KDEL-

transfected DCs is blocked by proteasome inhibitors, suggesting that the molecules are likely to enter the antigen processing pathway. We have shown that APCs, including DCs, containing the construct do not express CD80 or CD86 and are unable to stimulate either allospecific or peptide specific T cells both *in vitro* and *in vivo*. Indeed, antigen-reactive T cells are rendered anergic by encounter with CTLA4-KDEL expressing B cells and DCs. Furthermore, the anergic cells are capable of inhibiting a MLR indicating that they can act as regulatory cells. This suggests that the cells will be capable of mediating linked suppression, and may also regulate CD28 negative-T cells that can be present in patients.

While the cells were anergic as determined by proliferation, T cells did respond to CTLA4-KDEL transfected APC by an increase in IL4 and IL10 (in the case of B cells) secretion. Cultures contained a decreased amount of IFN γ and IL-12, indicating a Th2 skewing. Similar Th2 skewing has been reported for T cells rendered anergic by incubation with non professional antigen presenting cells or T cells, and has been reported *in vivo* following induction of anergy [109-111]. The expression of ICOS L and PD-L1 on DC transfected with CTLA4-KDEL was unaltered (Figure 36), and so this apparent Th2 bias may due to the unopposed (without CD28-CD80/86) costimulation through ICOS-ICOS L [112] and PD-PD-L1 [113] interactions, as has been previously described [112, 113]. This is in contrast what was seen when CTLA4-Ig was added to cultures containing control DC, which showed the expected reduction in IFN γ , IL4 and IL10 (data not shown), which may in DC be associated with upregulation of IDO (not seen in CTLA4-KDEL transfected cells).

Genetic modification of DCs as a strategy to induce tolerance has a number of advantages. DCs not only have the right antigen processing capacities for efficient presentation to T cells, but also traffick to the appropriate sites to encounter T cells. Our CTLA4-KDEL-expressing DCs have the potential advantage over drug-treated iDCs (which have been shown to be actively tolerogenic [9, 10]) in that the DCs can be activated to express high levels of MHC class II and will presumably show a normal homing pattern that allows efficient interaction with T cells. Furthermore, there is always the danger that drug-induced iDC will become activated *in vivo*, especially in sites with high concentrations of inflammatory cytokines. This approach also has the advantage over the administration of soluble proteins, such as CTLA4-Ig [126, 127], in that it is specific for the antigens expressed by the APCs and so will not induce generalised immunosuppression.

There is increasing evidence that CD80/86 is important for regulating immune responses, not only by interaction with CTLA4 on T cells but also by influencing regulatory cells. Thus CD80/86^{-/-} animals have a reduced number of CD4⁺CD25⁺ regulatory cells, and low levels of constitutive CD80/86 expression are necessary for the maintenance of regulatory cells [128]. In addition, lack of CD80/86-CD28 interactions can exacerbate autoimmunity [129]. However, CD80/86 expression on APC is not required for the induction of anergy (for example by anti-CD3 antibody [130]), and anergic cells can regulate immune responses *in vivo* [131]. It may be that in these settings CD80/86 effects are

operating in *trans*, possibly by CD80/86 expressed on T cells or (*in vivo*) by resident DC. Alternatively, different types of regulatory cells may have different requirements for co-stimulation.

One potential problem with the use of KDEL-tagged fusion proteins to form complexes in the ER is that this might stress the cell, leading to non-specific effects. In the case of human DCs, we were unable to demonstrate any upregulation of heat shock proteins. Nor was any upregulation of PERK (the ER stress response protein) and eIF-2 α observed. This was in contrast to cells transfected with intrabody (scFv-KDEL fusion protein [103]), which showed upregulation of PERK and eIF-2 α , possibly due to poor folding of the intrabody in the ER, with the subsequent activation of the ER stress pathway. The use of a surface ligand, which is normally expressed in the ER, may be an advantage in this respect. However, in contrast to the situation in human, there was transient upregulation of PERK in murine DCs, associated with phosphorylation of eIF-2 α , which is known to decrease protein synthesis [102, 119]. This may explain the transient downregulation of MHC class II and CD11c expression in these cells.

Cross-linking of CD80/86 on DC by CTLA4-Ig results in upregulation of the IDO enzyme resulting in increased tryptophan metabolism [132]. However, the inhibition of proliferation by CTLA4-KDEL transfected DC was independent of IDO, as determined by the failure of 1-MT to block the inhibition and the lack of IDO mRNA in these cells during a MLR. This indicates that binding of CD80/86 by CTLA4-KDEL in the ER does not activate IDO transcription.

In summary, we have demonstrated in a human system a new strategy for inducing immunological tolerance. Our findings support the possibility of clinical therapy either by administration of *ex-vivo* CTLA4-KDEL transfected APCs or by gene transfer of the construct to APCs *in vivo*. The ability of T cells anergised by contact with CTLA4-KDEL-transfected APCs to regulate the response of other T cells indicates that they may be effective in settings when only some of the antigens are expressed on DCs, or when only a proportion of the antigen-specific T cells has contact with the CTLA4-KDEL-expressing DCs. This approach could find application either in the context of transplantation, in which control of allospecific T cells is required or autoimmunity when tolerance to particular antigens is needed. The development of similar fusion proteins may also be used to block the expression of other co-stimulatory molecules, thus allowing more a subtle modulation of immune responses.

It will be understood that the invention has been described by way of example only and modifications may be made whilst remaining within the scope and spirit of the invention.

SEQUENCE LISTING

SEQ ID NO: 1.

KDEL

SEQ ID NO: 2.

HDEL

SEQ ID NO: 3.

DDEL

SEQ ID NO: 4.

ADEL

SEQ ID NO: 5.

SDEL

SEQ ID NO: 6.

QDEL

SEQ ID NO: 7.

KEDL

SEQ ID NO: 8.

KDEI

SEQ ID NO: 9.

[KRHQSA] - [DENQ] - E - L

SEQ ID NO: 10.

HTEL

SEQ ID NO: 11.

HVEL

SEQ ID NO: 12.

HIEL

SEQ ID NO: 13.

KKXX

SEQ ID NO: 14.

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SEQ ID NO: 15.

MYPPPY

SEQ ID NO: 16.

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SEQ ID NO: 17.

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SEQ ID NO: 18.

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SEQ ID NO: 19.

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SEQ ID NO: 20.

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SEQ ID NO: 21.

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1. A protein comprising: (a) a first sequence which causes the protein to be retained in the endoplasmic reticulum; and (b) a second sequence which can bind to a co-stimulatory ligand of an antigen presenting cell.
2. The protein of claim 1, wherein the first sequence comprises KDEL.
3. The protein of claim 1 or claim 2, wherein the co-stimulatory ligand is CD80, CD86, B7-H1, PD-L2, B7-H2 or B7-H3.
4. The protein of claim 3, wherein the second sequence is derived from CD152, CD28, PD-1 or ICOS.
5. The protein of any one of claims 1 to 3, wherein the second sequence is an anti-ligand antibody.
6. Nucleic acid which encodes a protein according to any preceding claim.
7. A vector comprising the nucleic acid of claim 6.
8. A cell containing a protein according to any one of claims 1 to 5, a nucleic acid of claim 6, or a vector of claim 7.
9. The cell of claim 8, wherein the cell is an antigen presenting cell and/or a dendritic cell.
10. A method of treating a patient, comprising the step of delivering to the patient a protein according to any one of claims 1 to 5, a nucleic acid of claim 6, a vector of claim 7, or a cell of claim 8 or claim 9.
11. A protein according to any one of claims 1 to 5, a nucleic acid of claim 6, a vector of claim 7, or a cell of claim 8 or claim 9, for use in medicine.
12. The use of a protein according to any one of claims 1 to 5, a nucleic acid of claim 6, a vector of claim 7, or a cell of claim 8 or claim 9, in the manufacture of a medicament for immunosuppression, immunomodulation, immunostimulation and/or tolerance induction.
13. The use of claim 12, wherein the medicament is for treating autoimmune disease, hypersensitivity reactions or transplant rejection *e.g.* for treating lupus, psoriasis vulgaris, asthma, rheumatoid arthritis, or an allergy.
14. A protein comprising: (a) a first sequence which causes the protein to be retained in the endoplasmic reticulum; and (b) a second sequence from a cell surface receptor.
15. A method for investigating a cell-surface protein, comprising the steps of: (a) expressing a polypeptide in one or more cell types, wherein the polypeptide comprises (i) at least the extracellular binding domain of the cell-surface protein, and (ii) a sequence which causes the polypeptide to be retained in the endoplasmic reticulum; and (b) comparing the phenotype(s) of the one or more cell types with control cells which do not express the polypeptide.
16. A method for preparing a genetically-modified dendritic cell, comprising the steps of: (a) genetically modifying a monocyte; and (b) differentiating the monocyte into a dendritic cell.

FIGURE 1

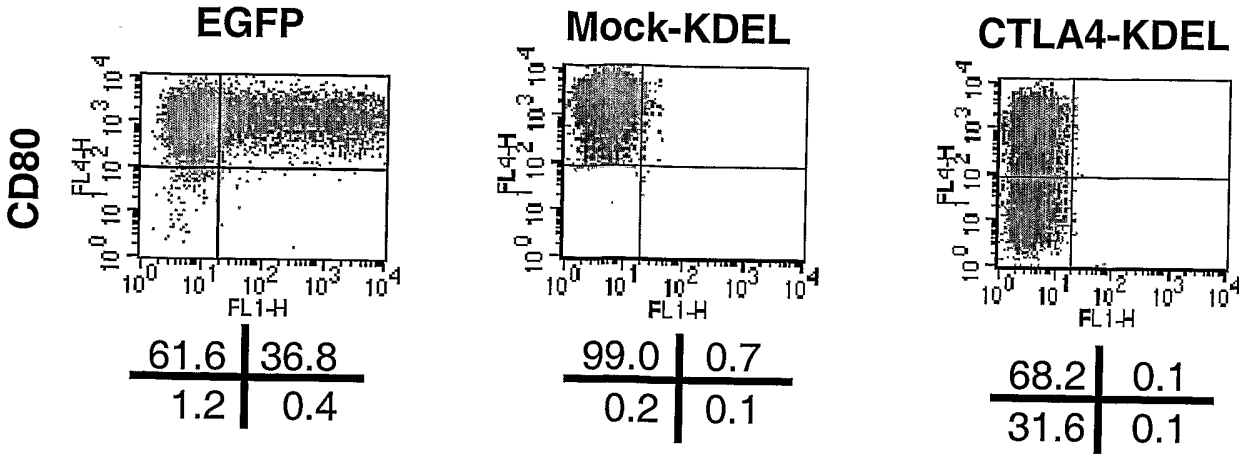


FIGURE 2A

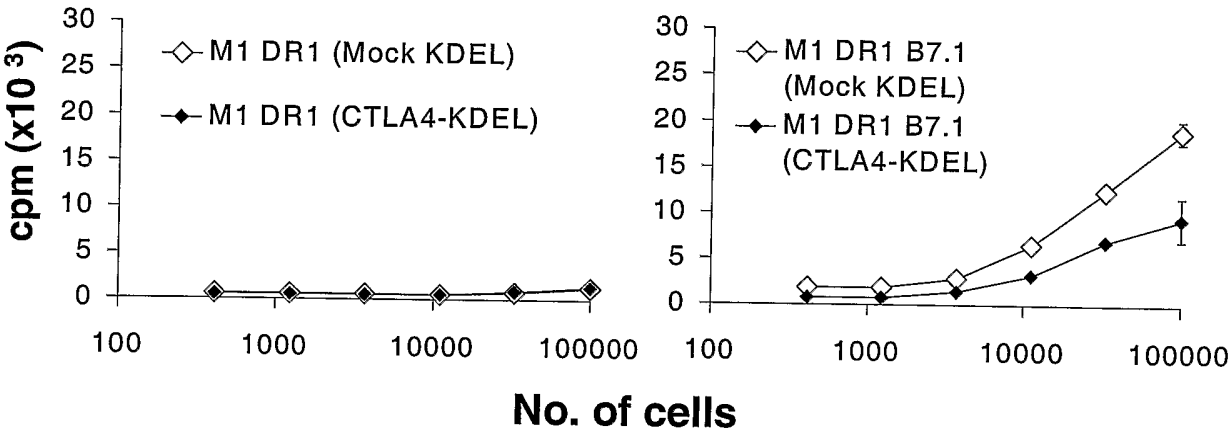


FIGURE 2B

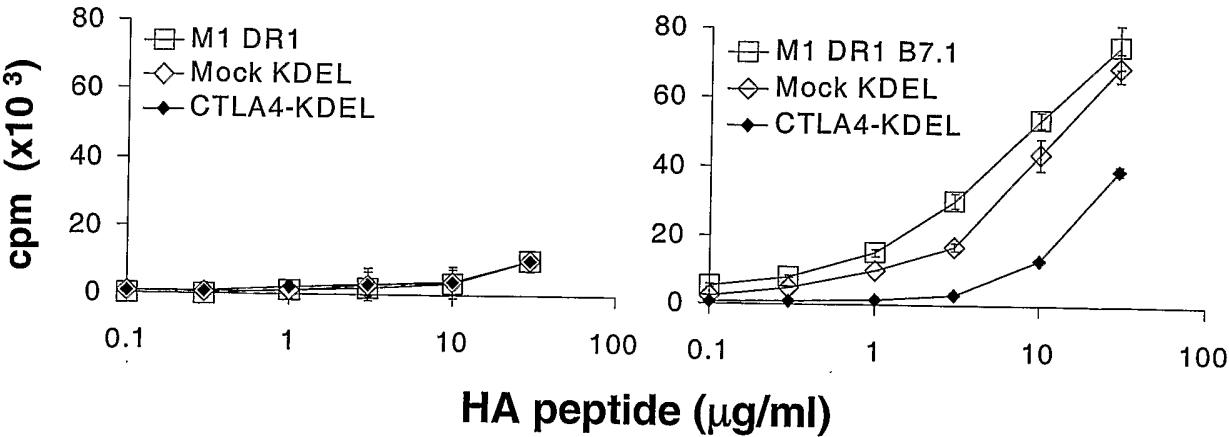


FIGURE 3

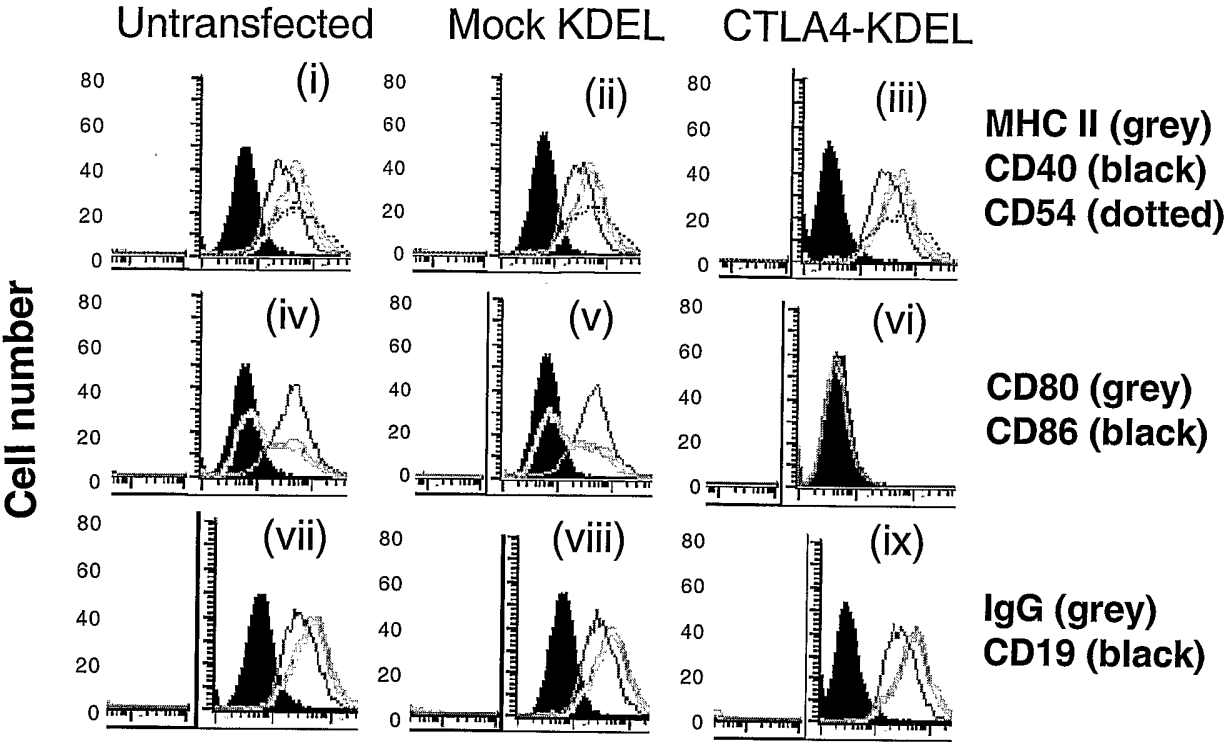
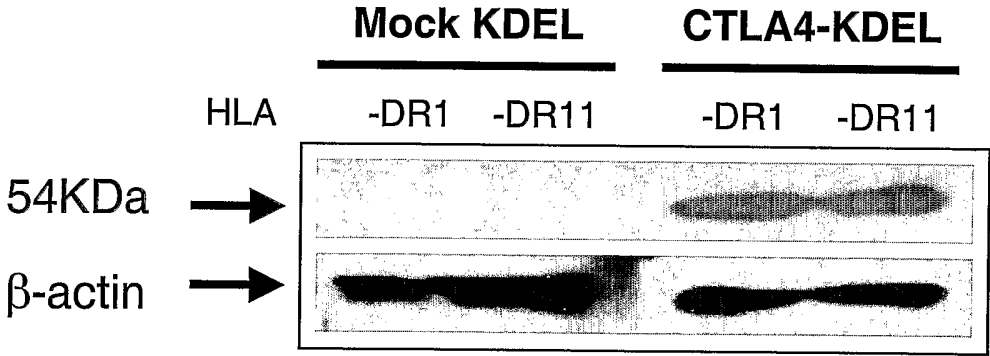
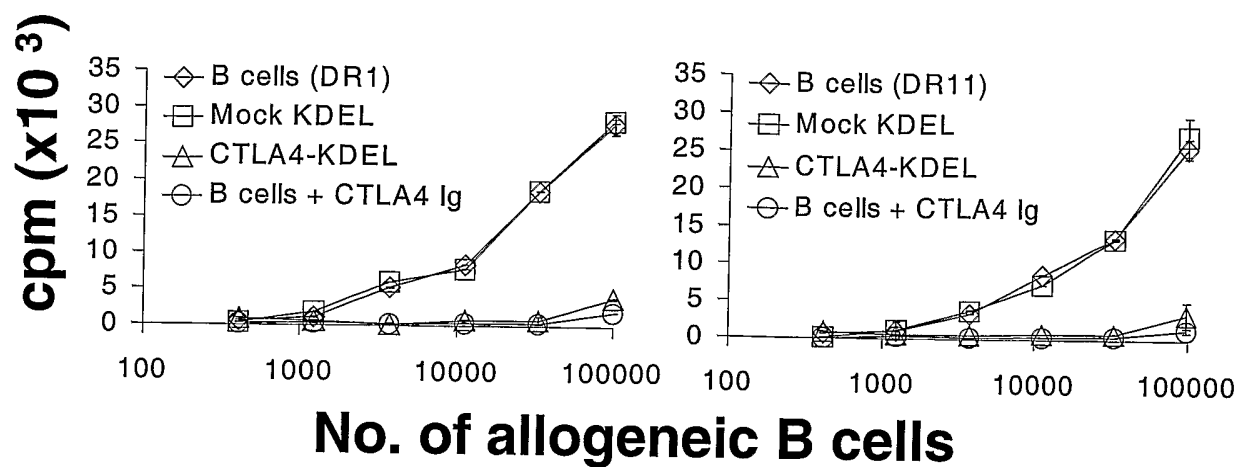
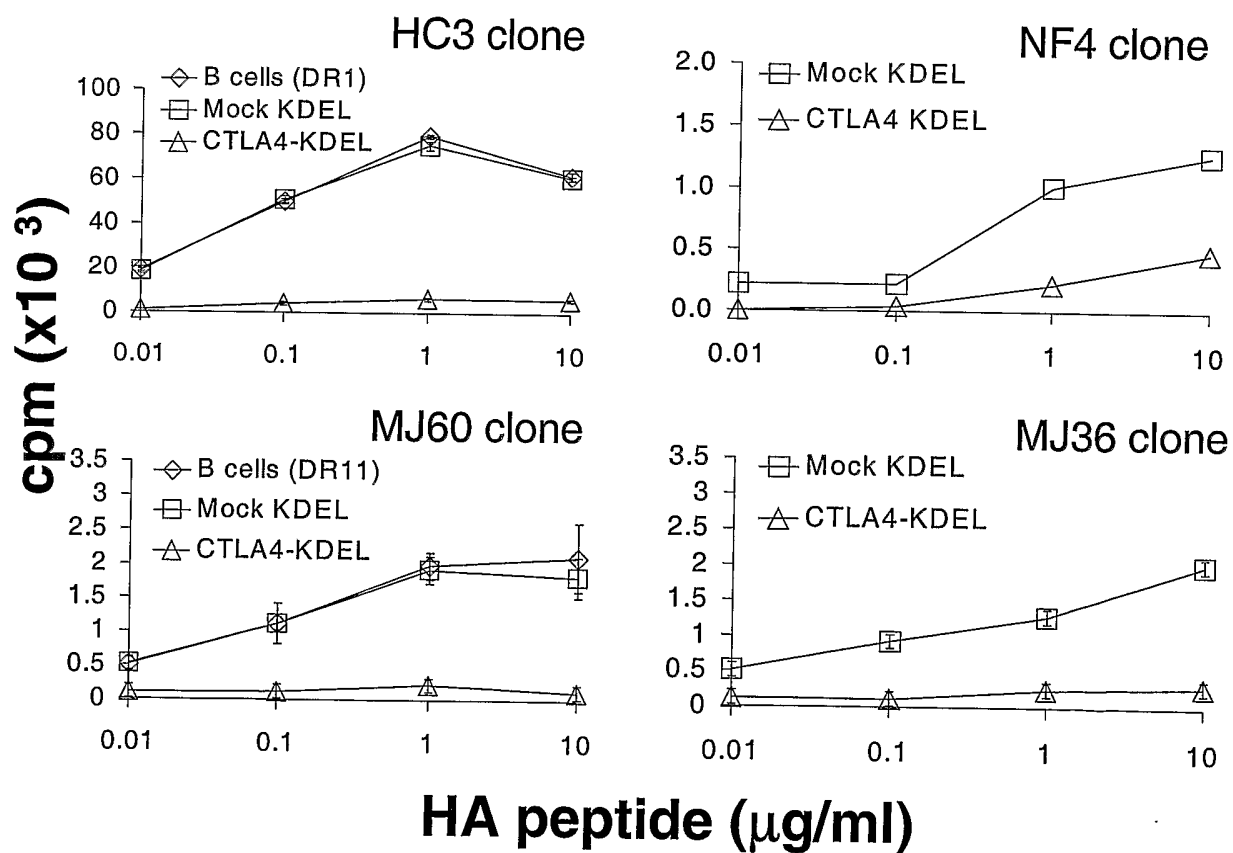


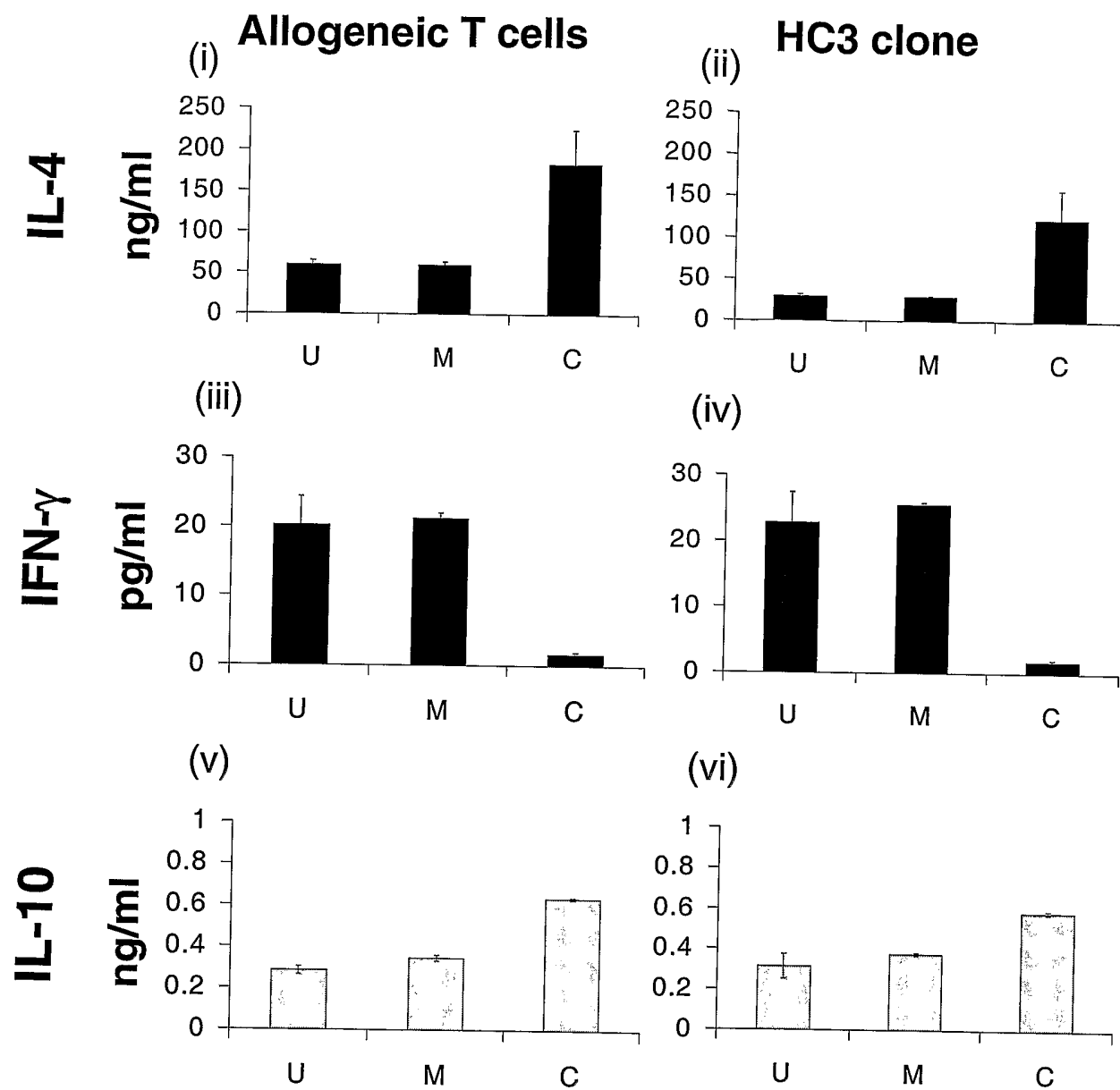
FIGURE 4



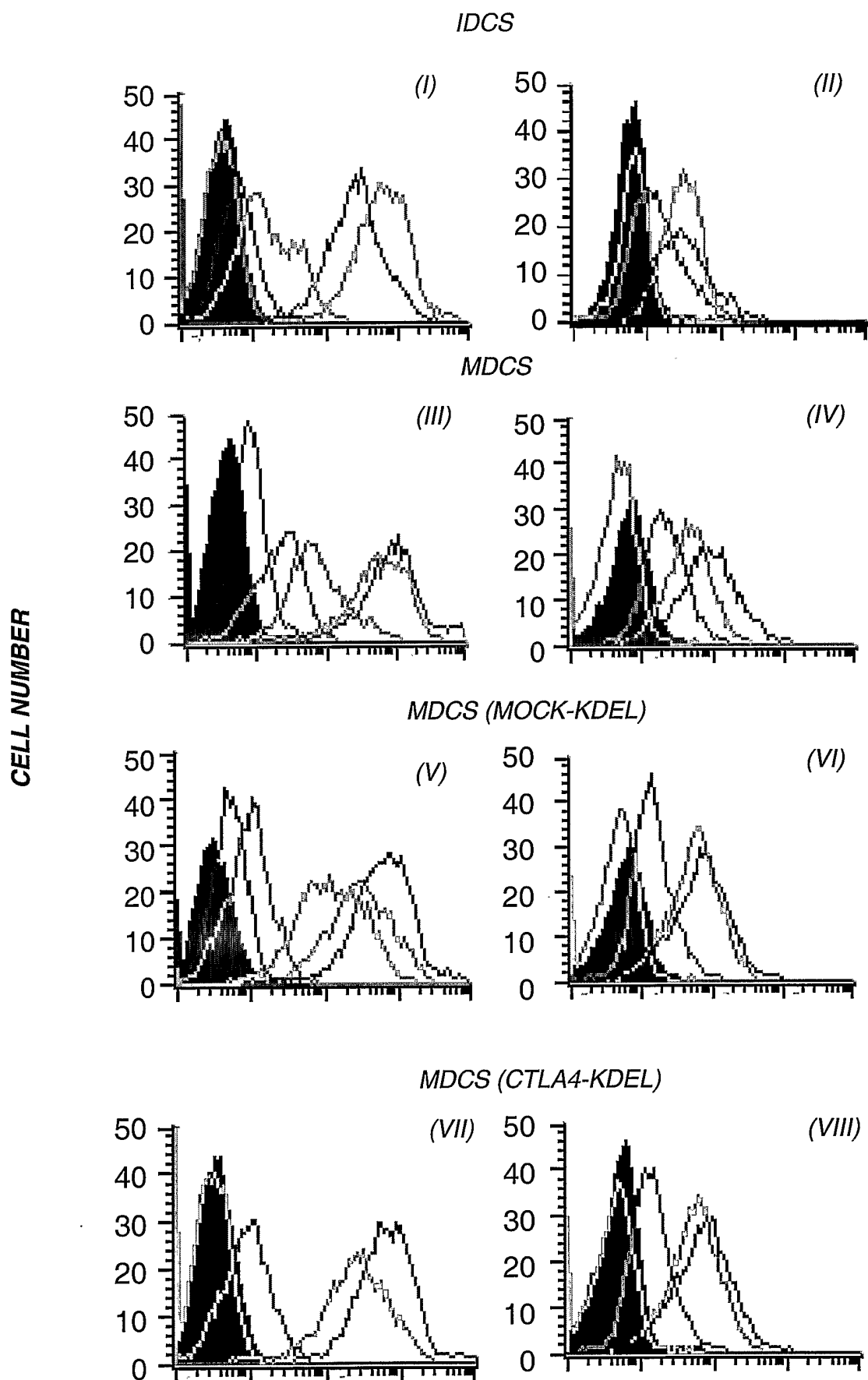
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FIGURE 5**FIGURE 6**

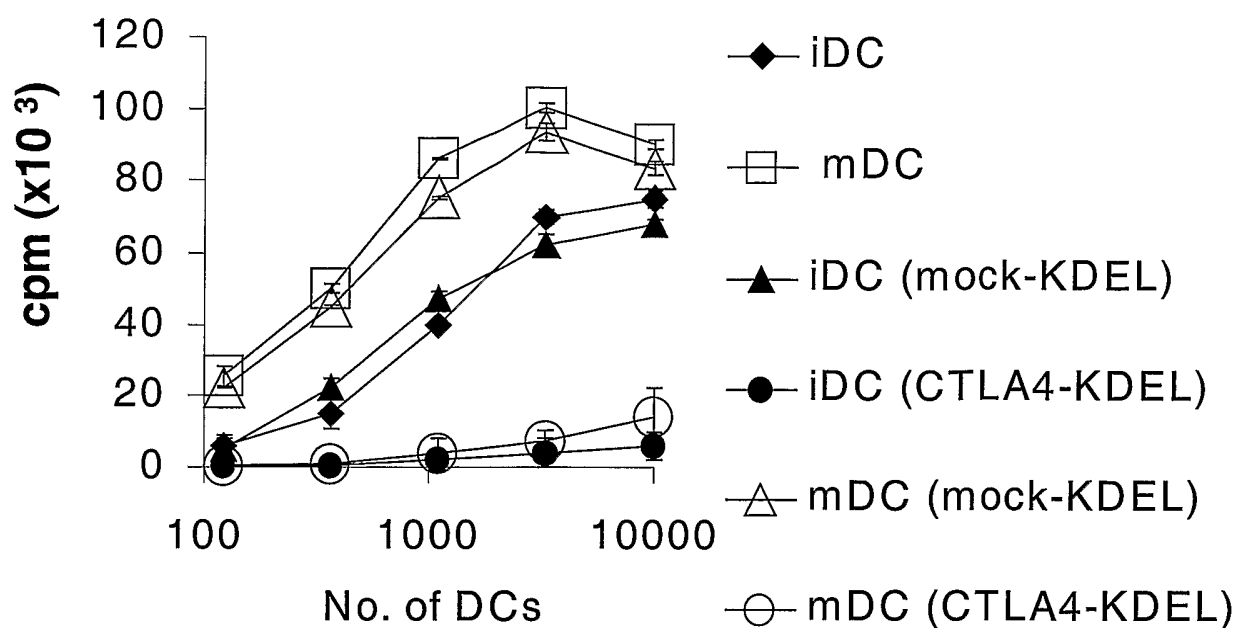
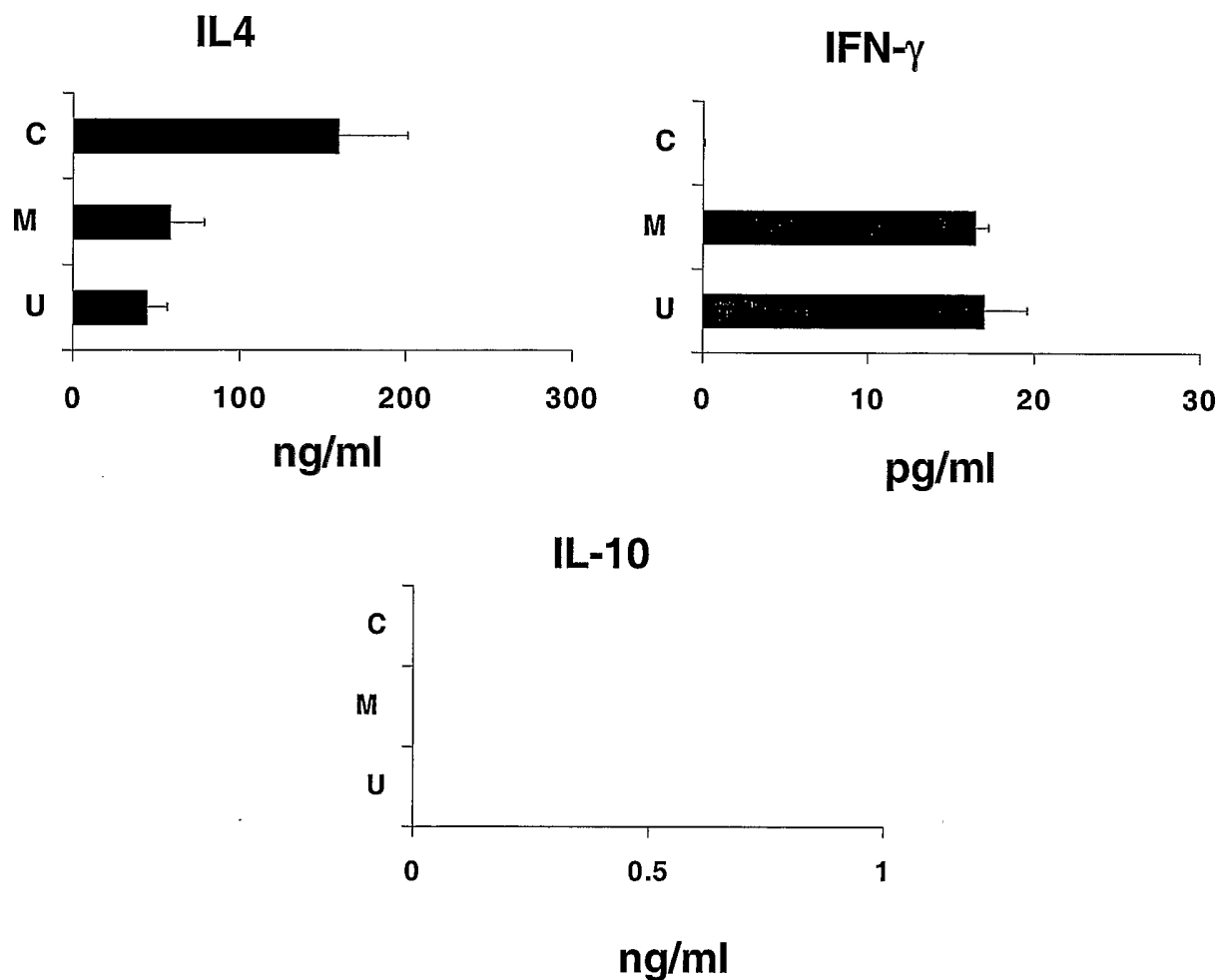
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FIGURE 7

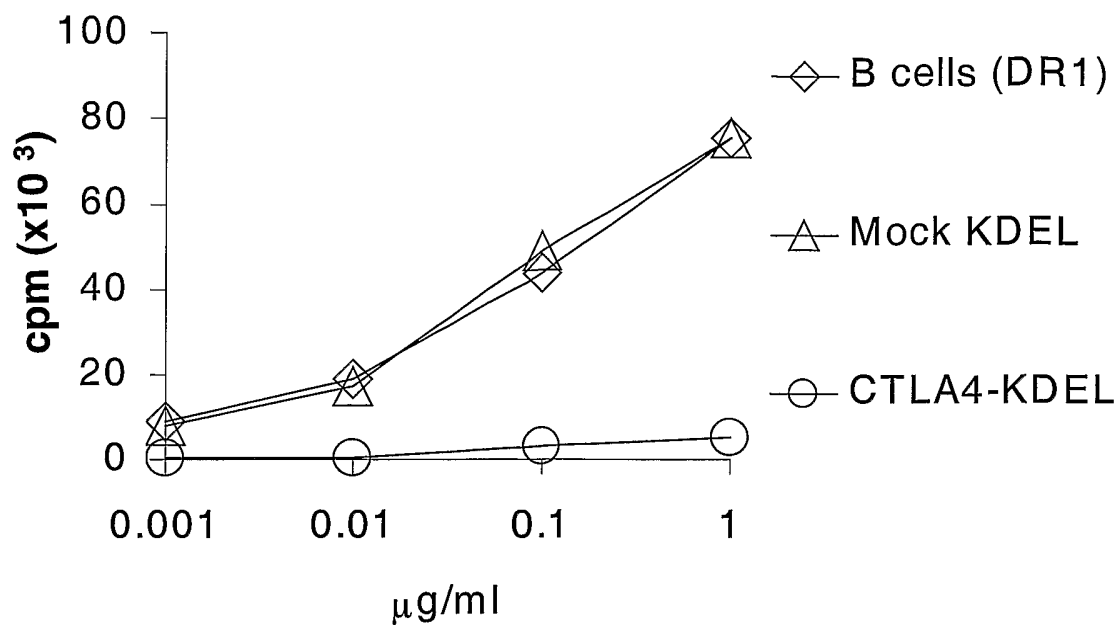
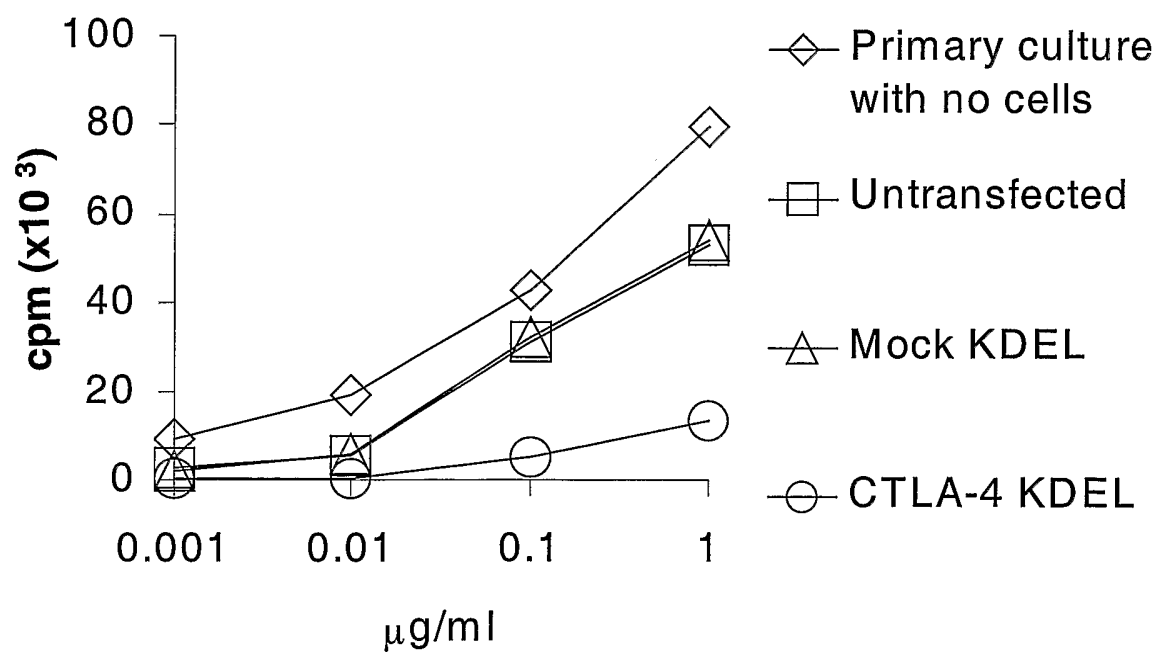
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FIGURE 8

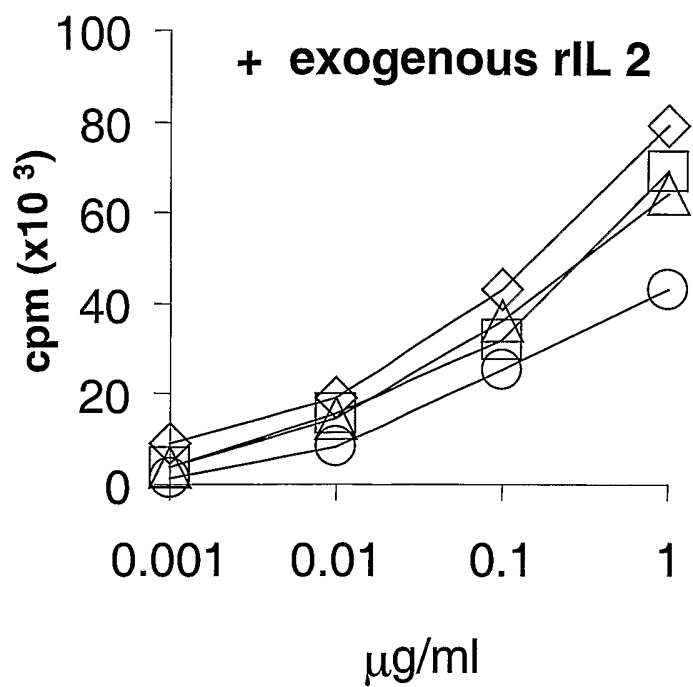
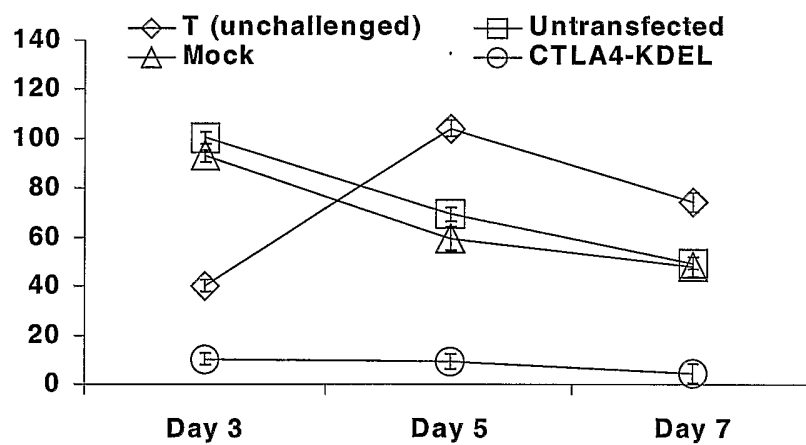
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FIGURE 9**FIGURE 10**

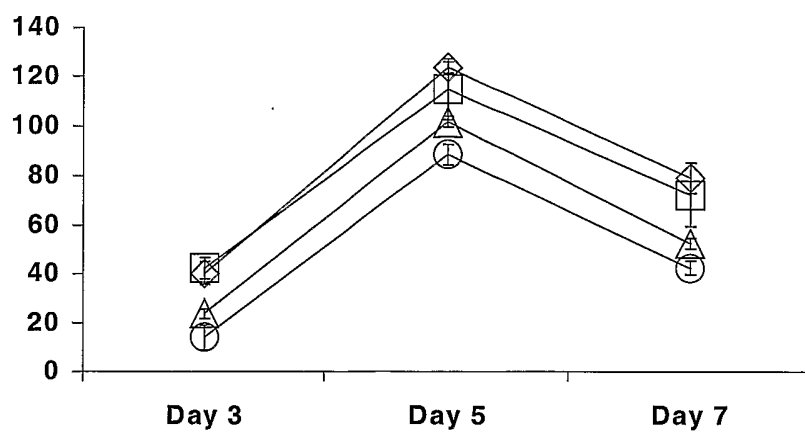
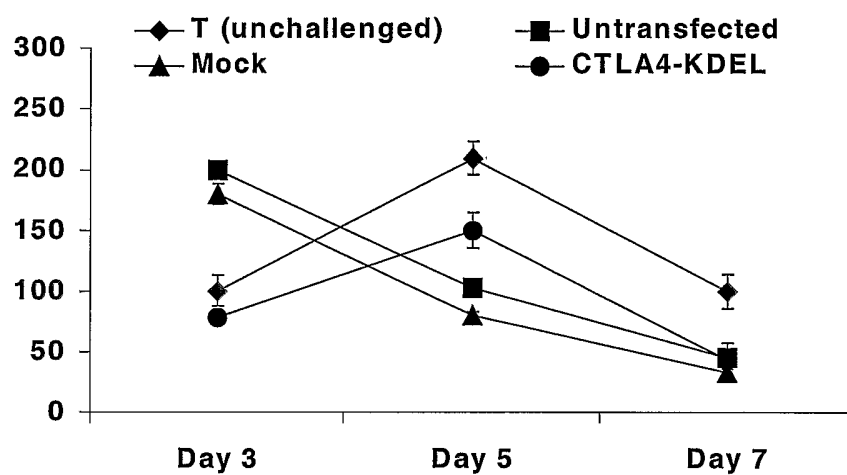
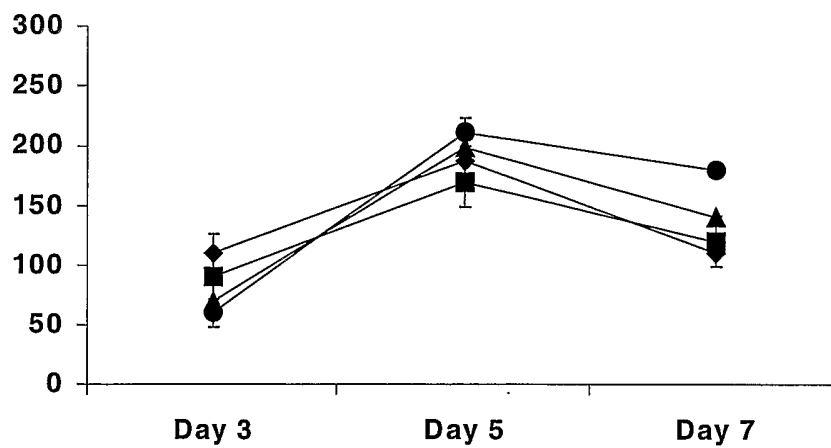
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FIGURE 11A**FIGURE 11B**

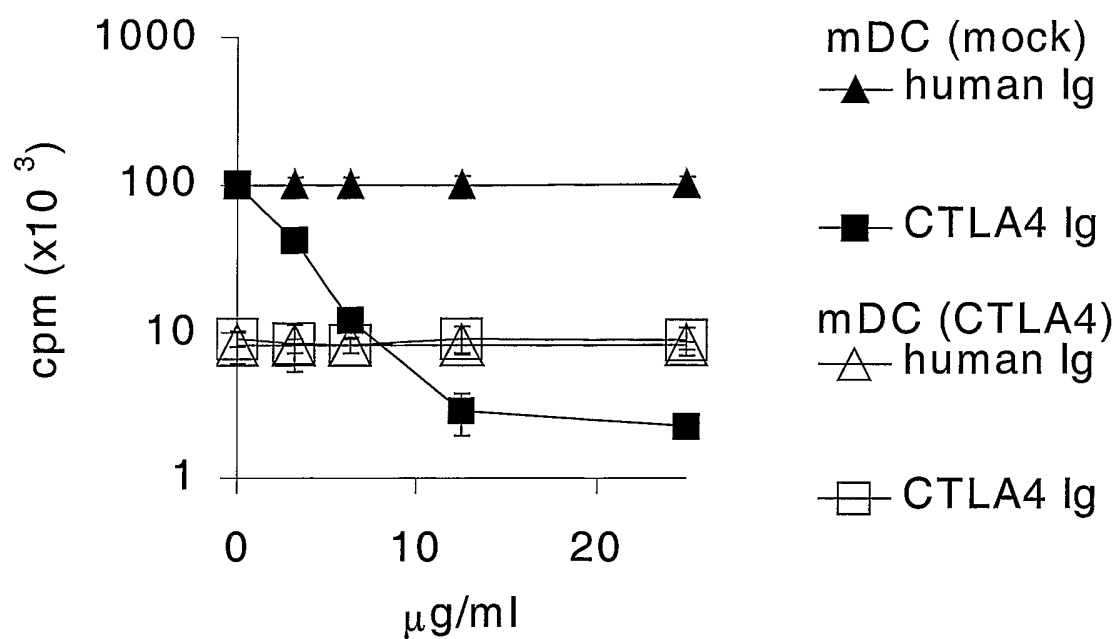
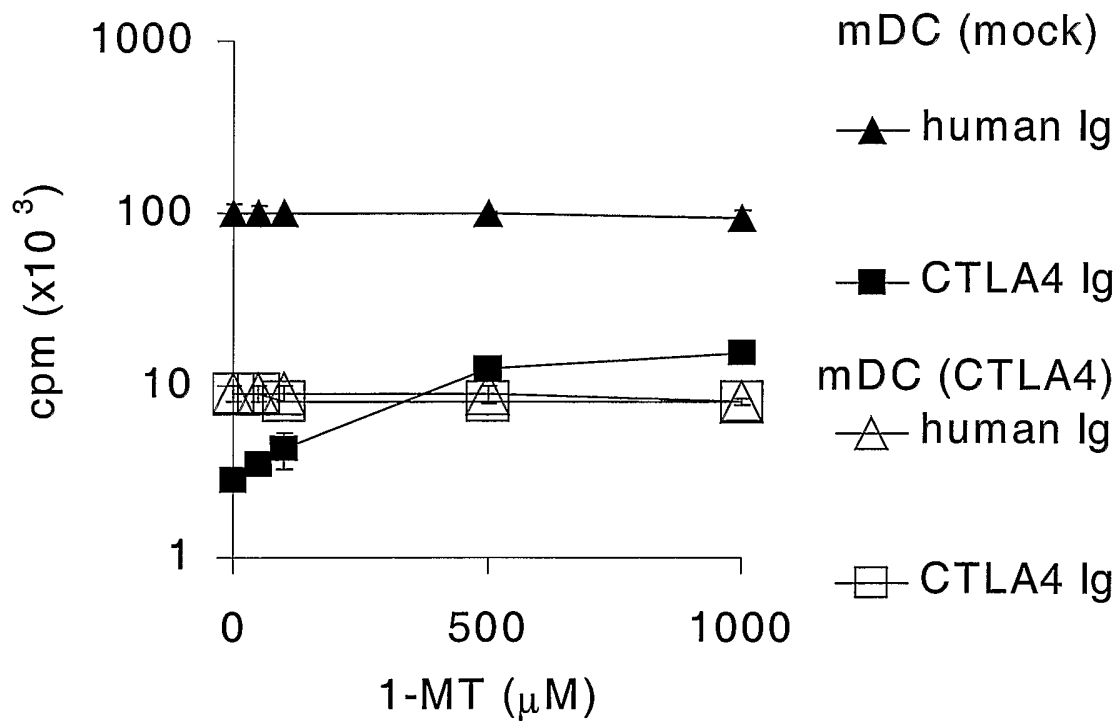
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FIGURE 11C**FIGURE 12A**

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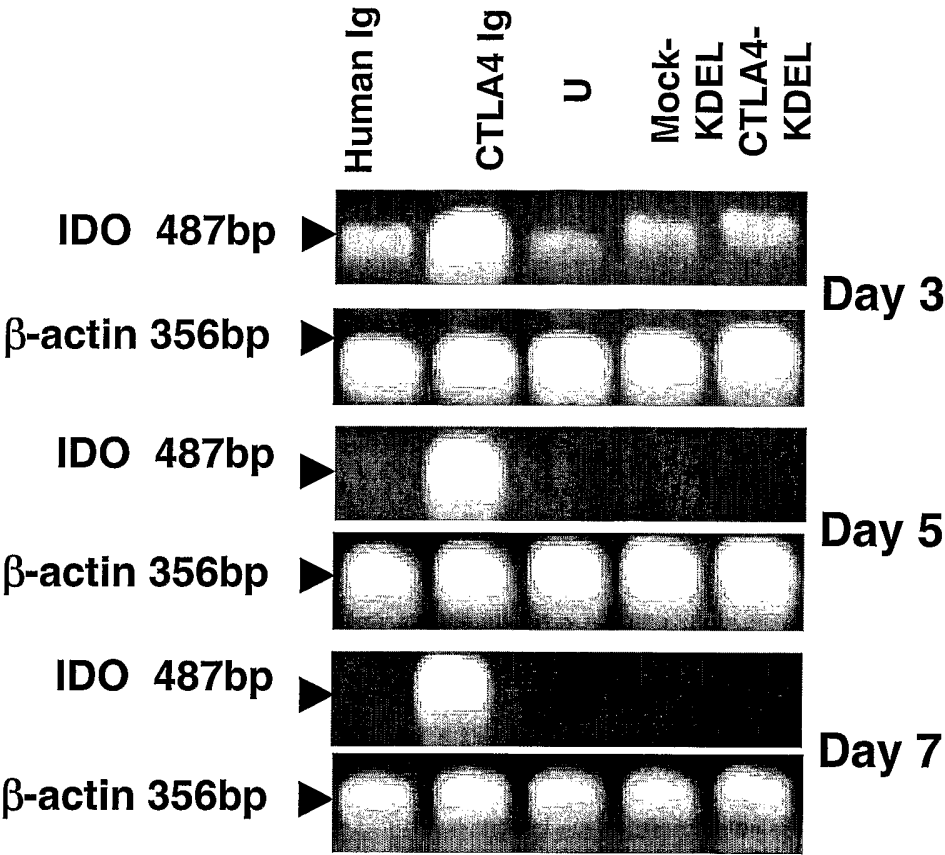
FIGURE 12B**FIGURE 12C****FIGURE 12D**

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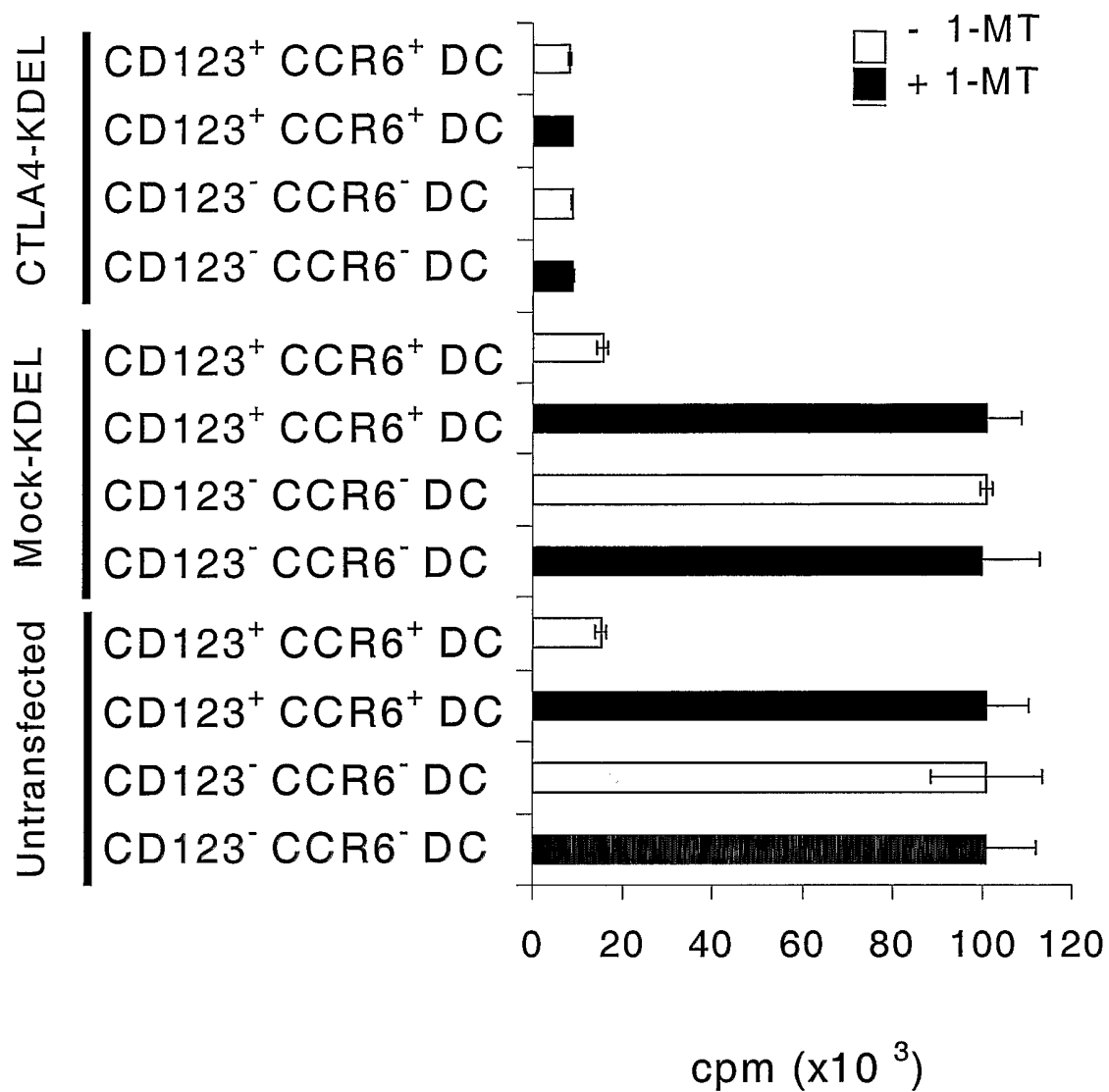
FIGURE 13**FIGURE 14**

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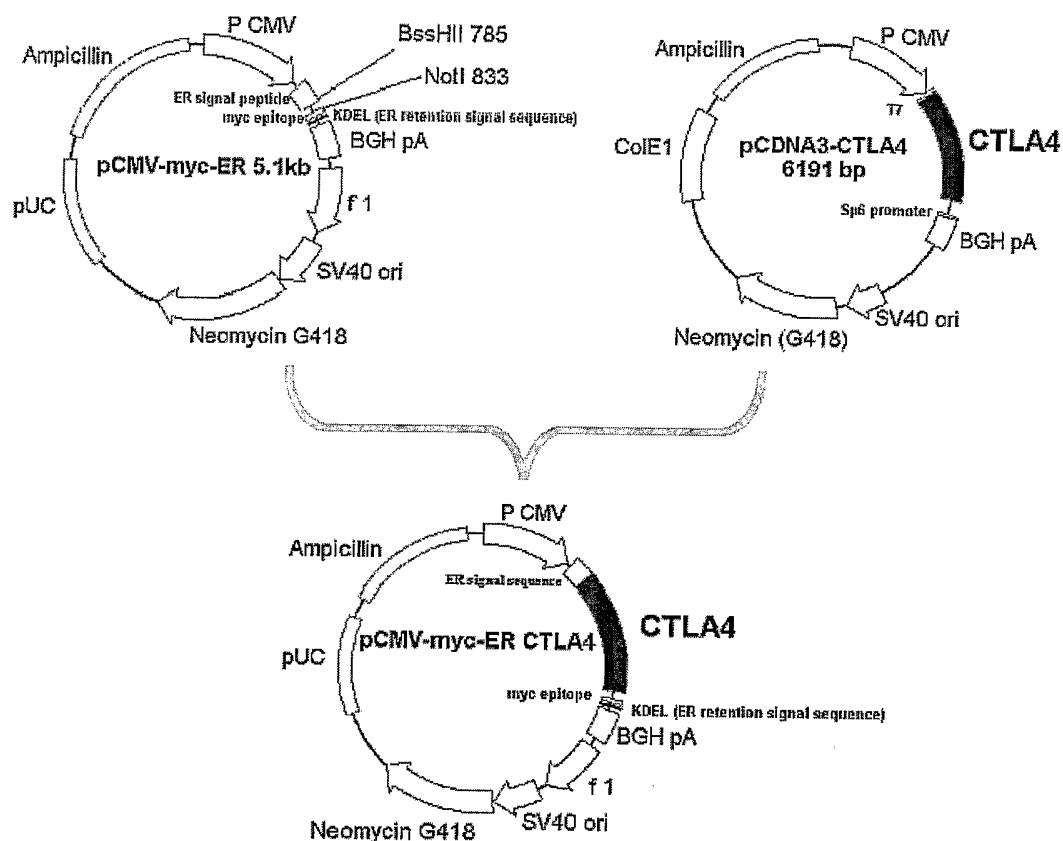
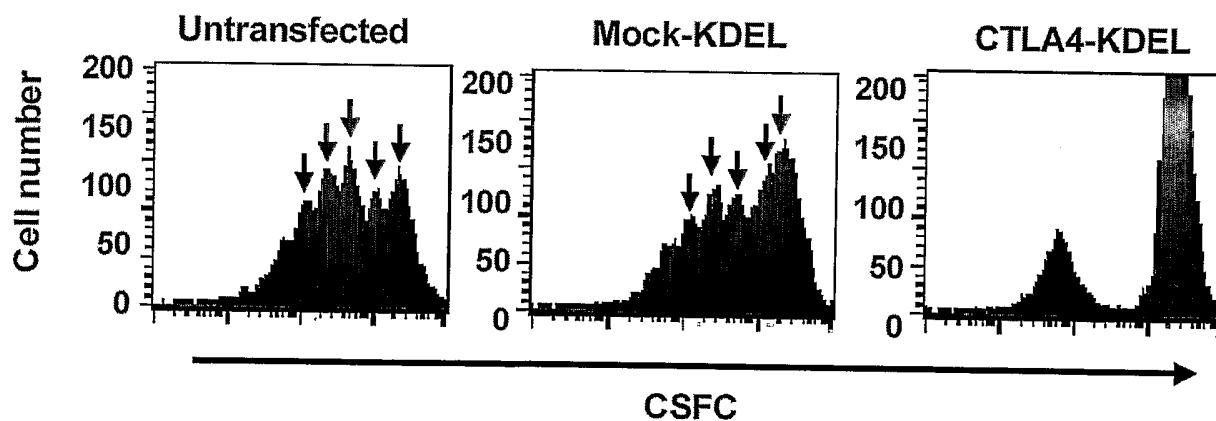
FIGURE 15



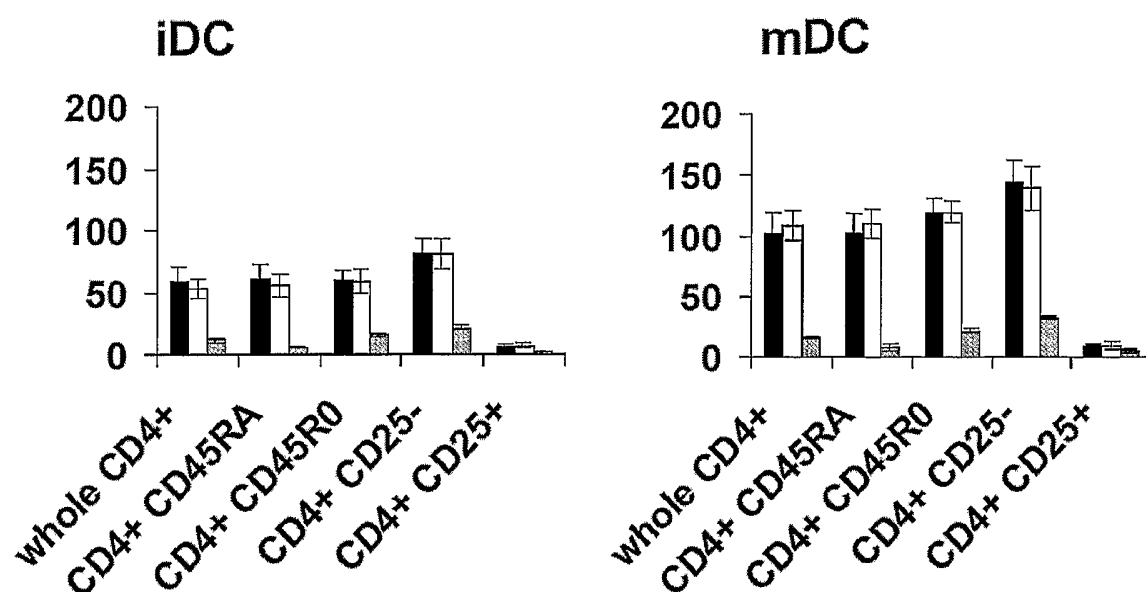
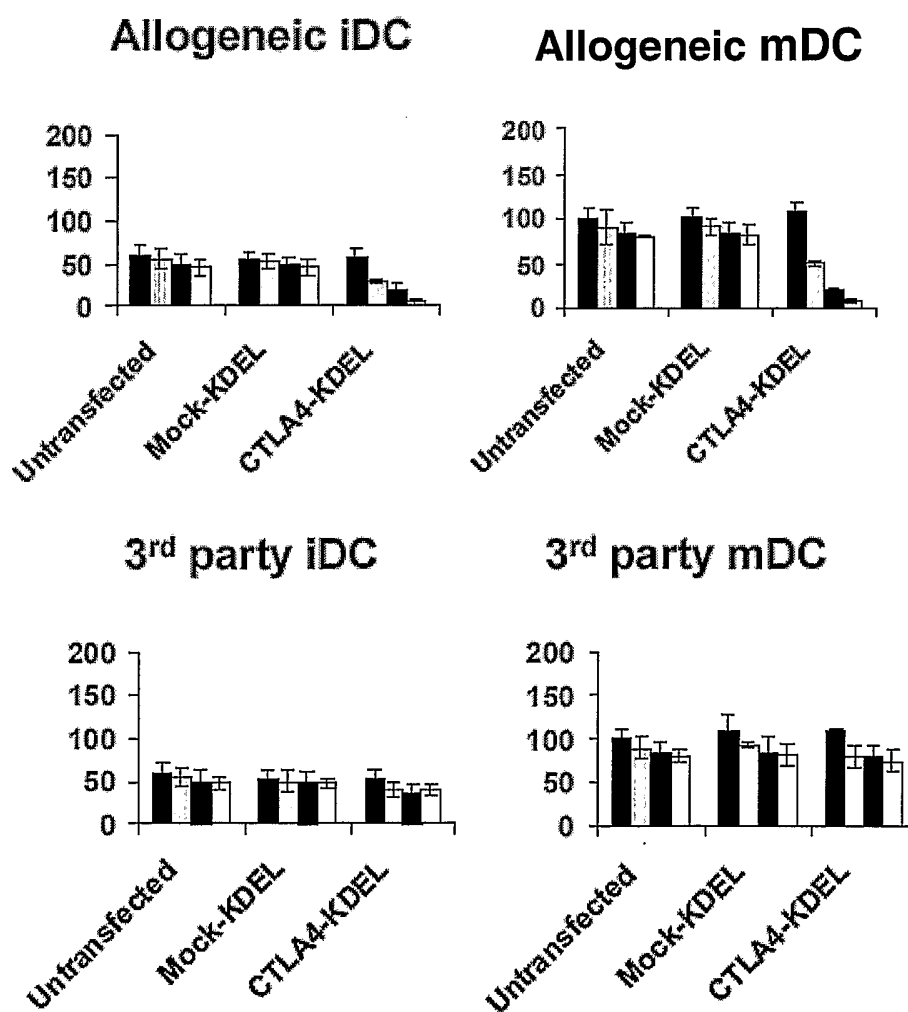
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FIGURE 16

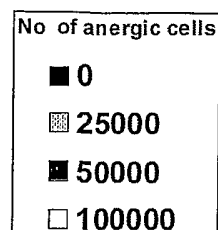
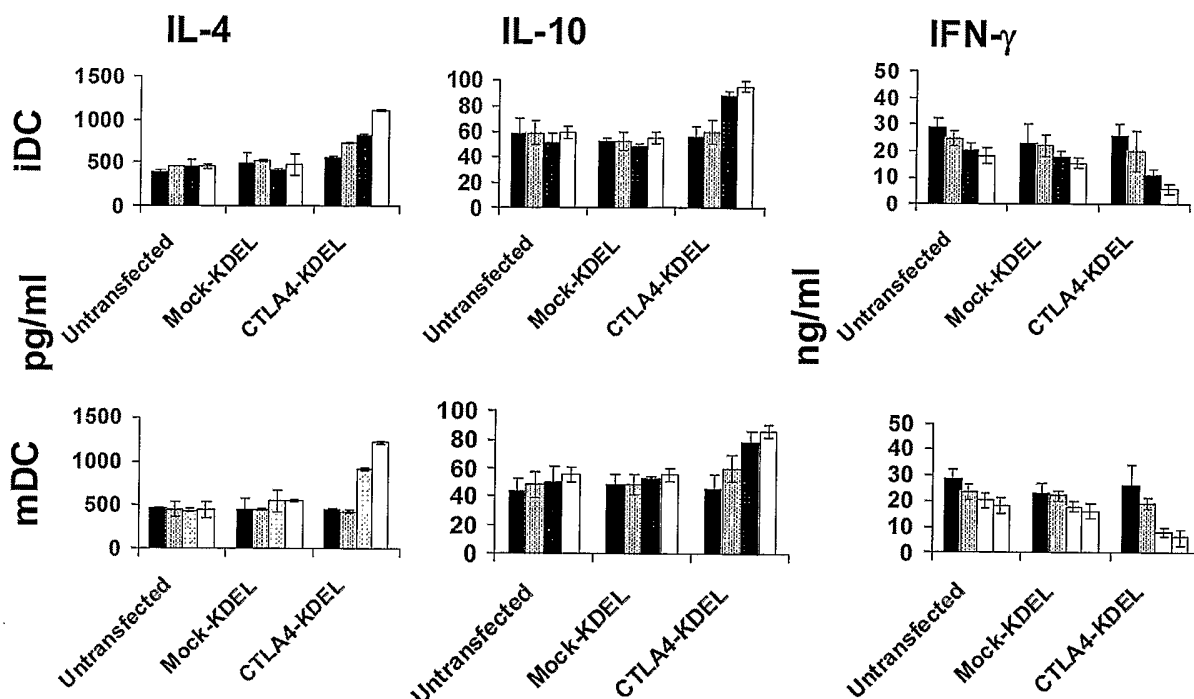
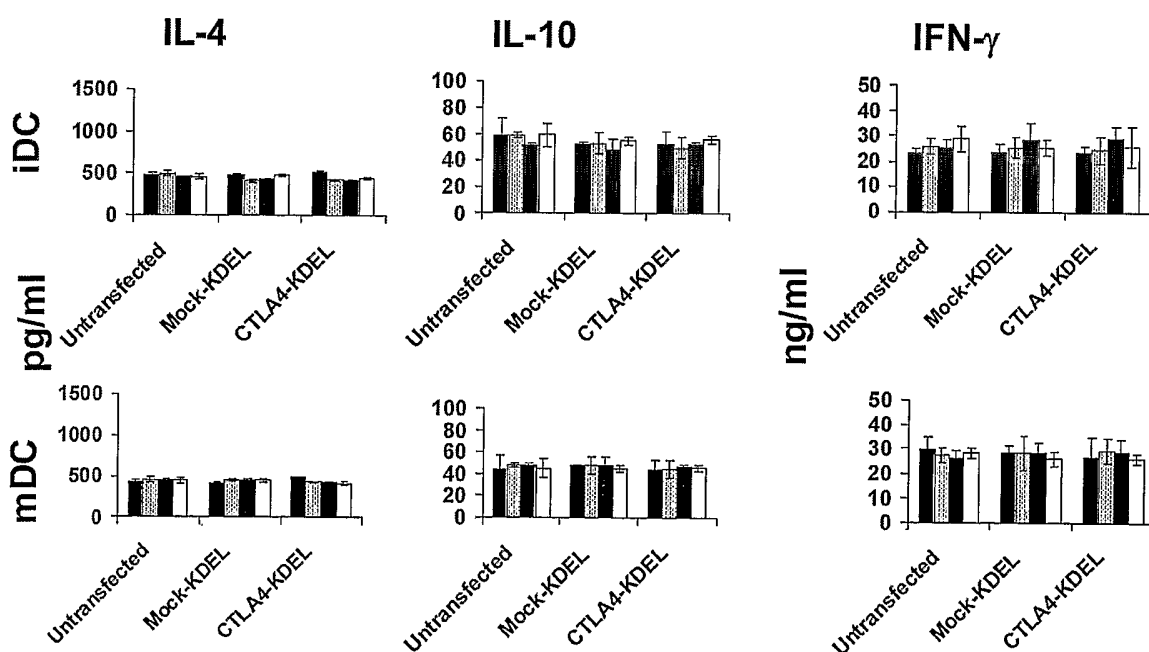
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FIGURE 17**FIGURE 18**

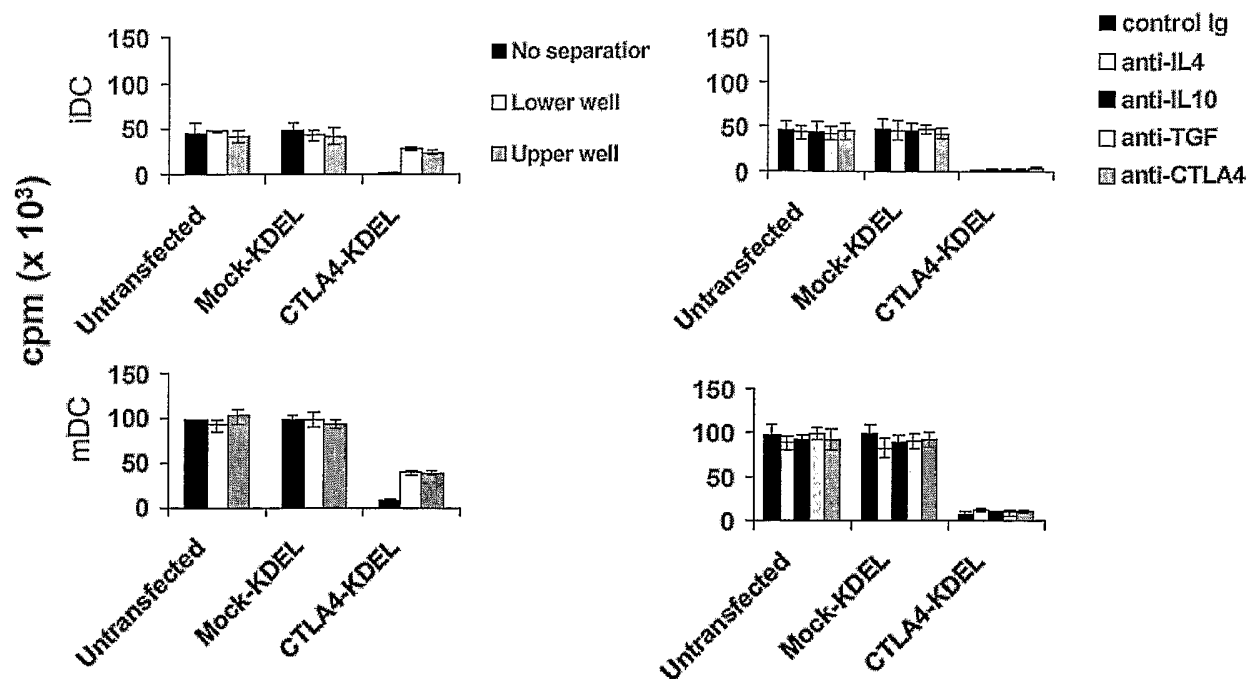
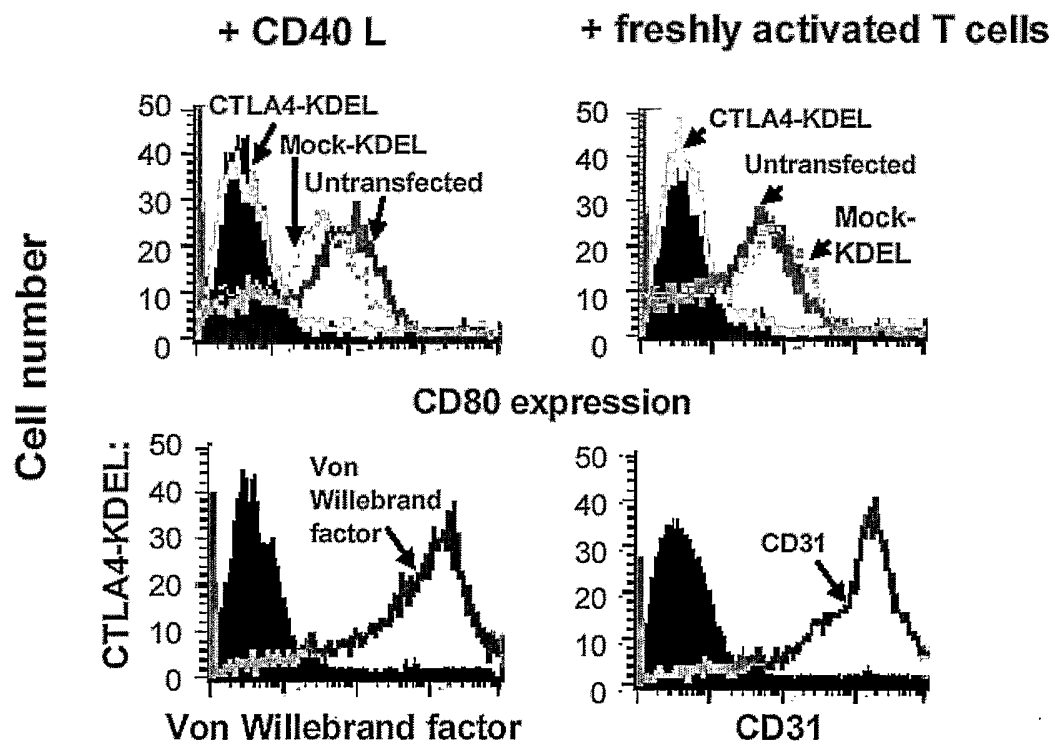
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FIGURE 19**FIGURE 20**

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FIGURE 21**Allogeneic response:****3rd party response:**

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FIGURE 22**FIGURE 23**

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FIGURE 24

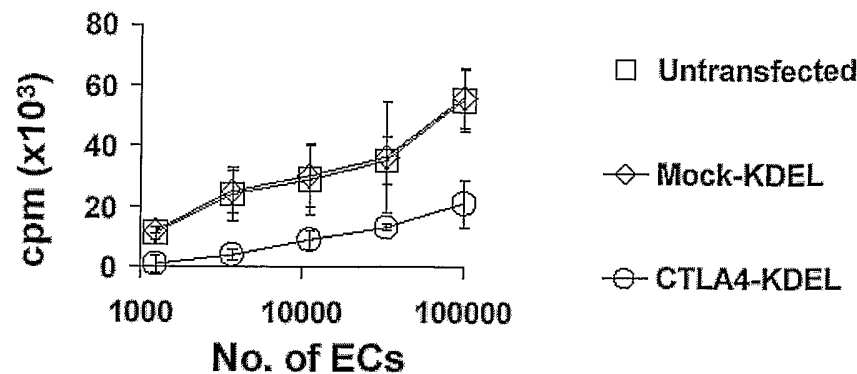


FIGURE 25

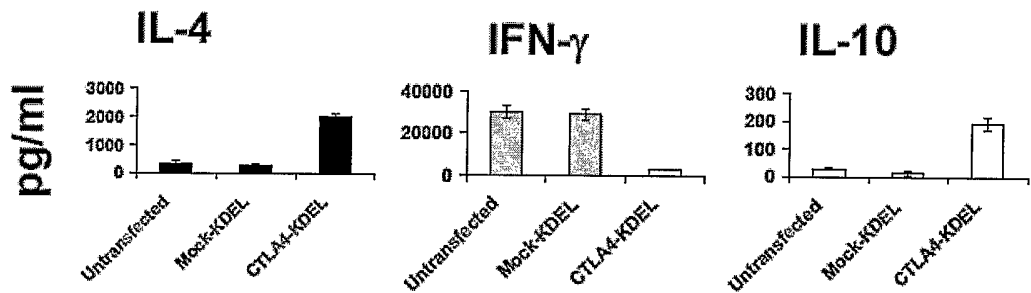


FIGURE 26

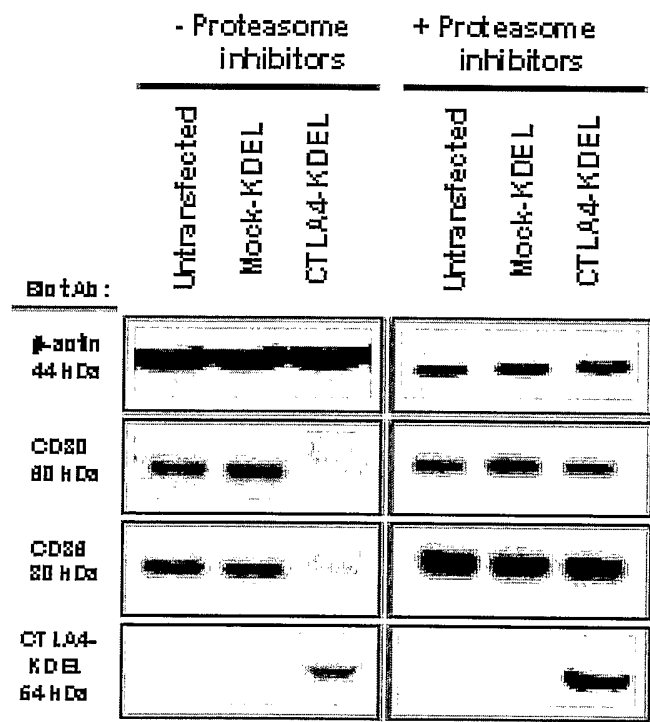


FIGURE 27

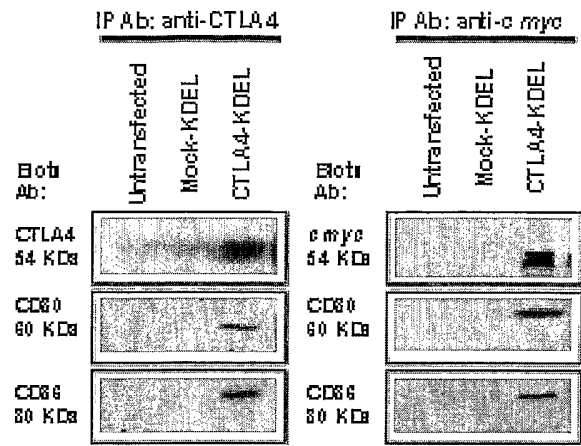


FIGURE 29

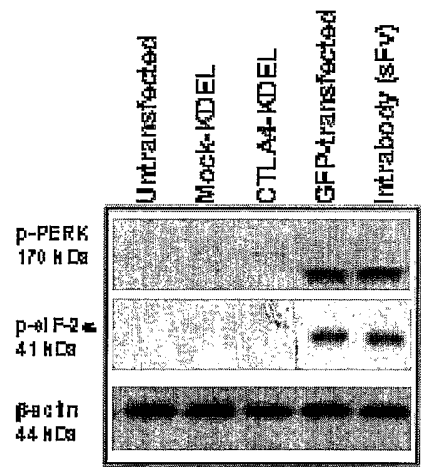
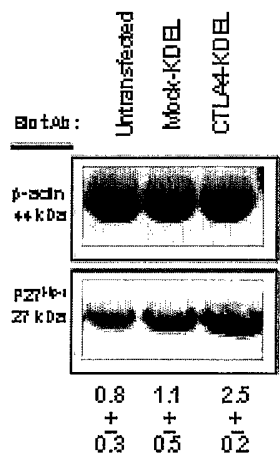


FIGURE 31



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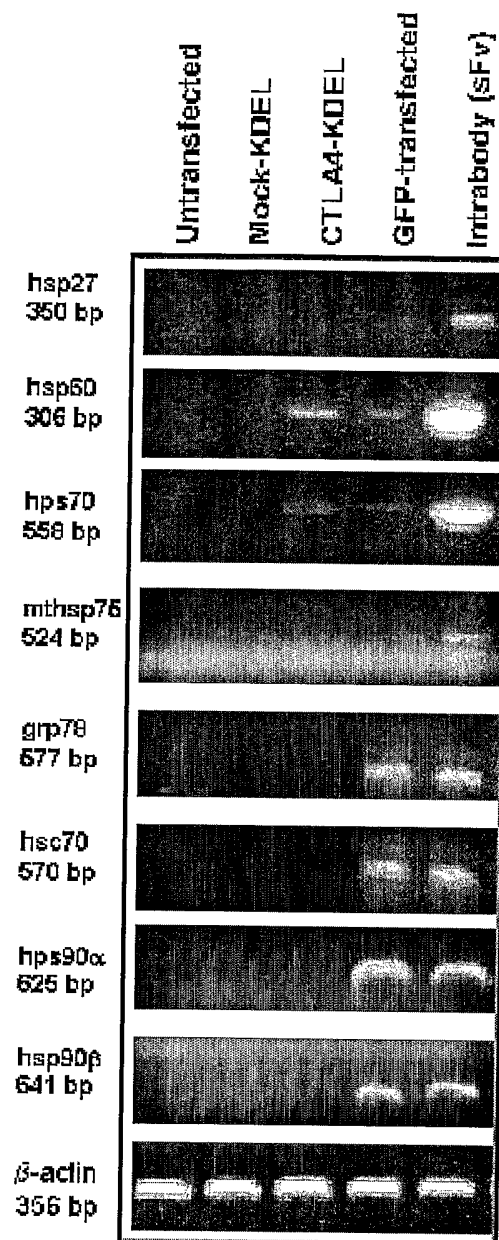
FIGURE 28

FIGURE 30

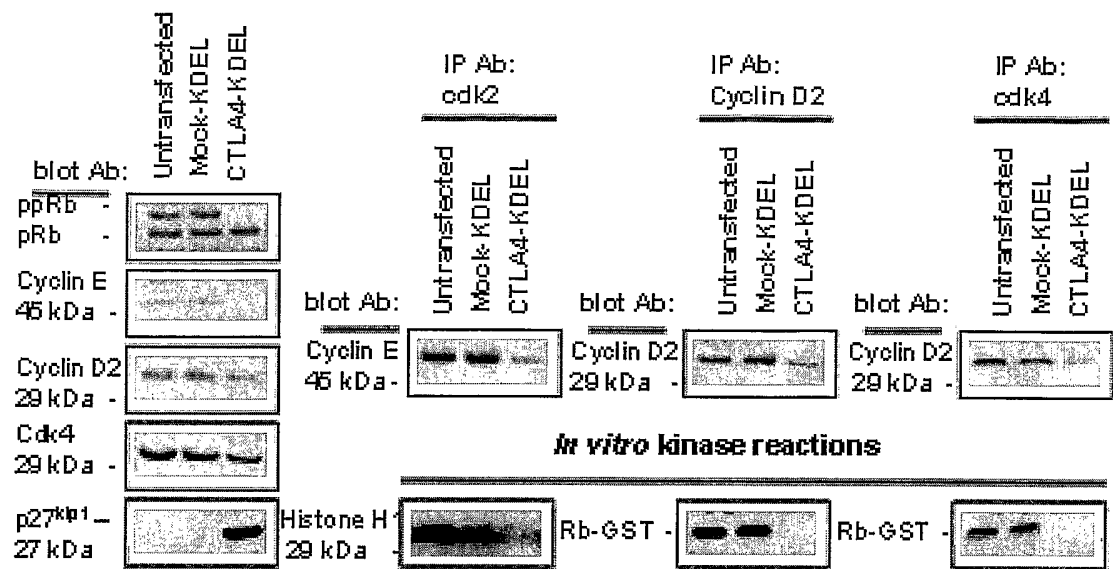


FIGURE 32

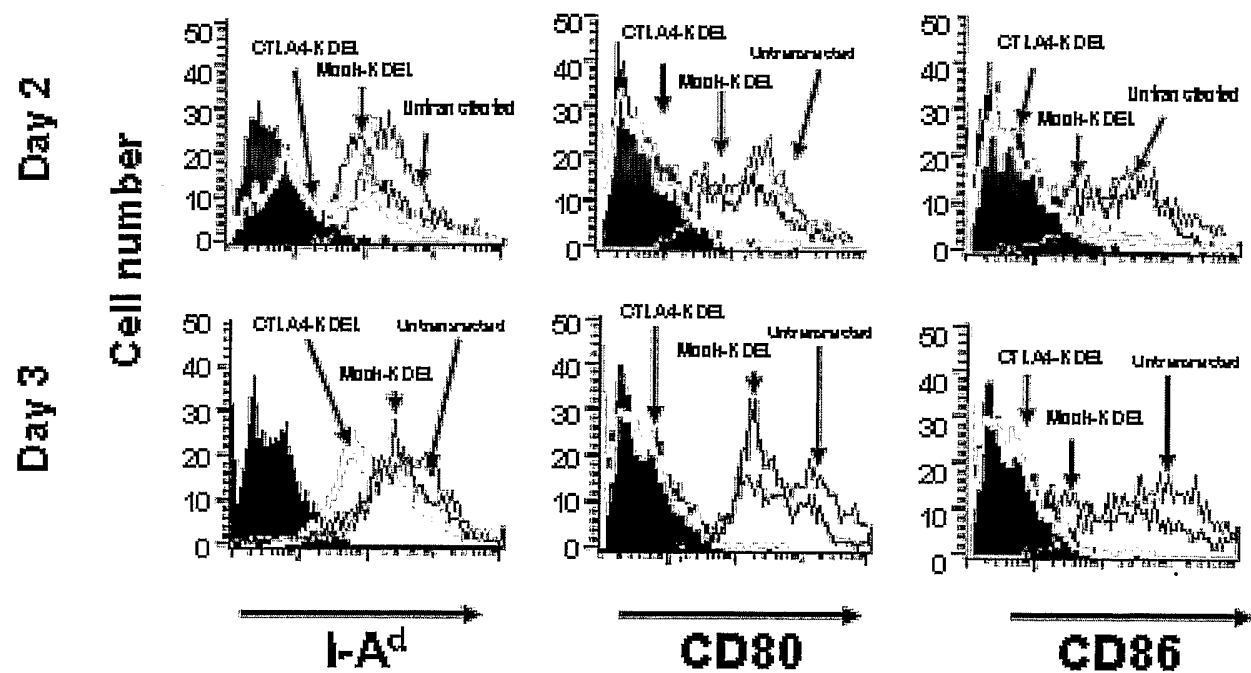


FIGURE 33

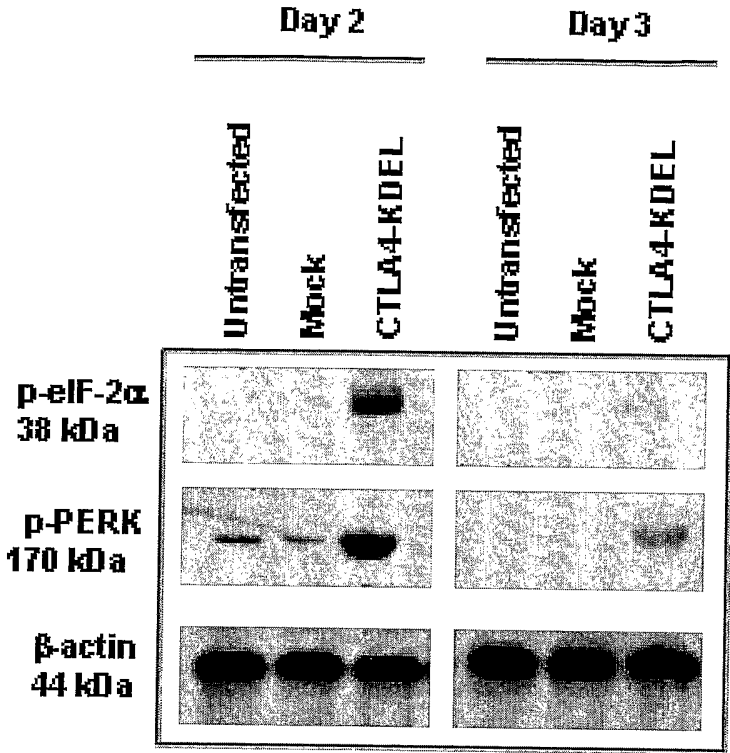
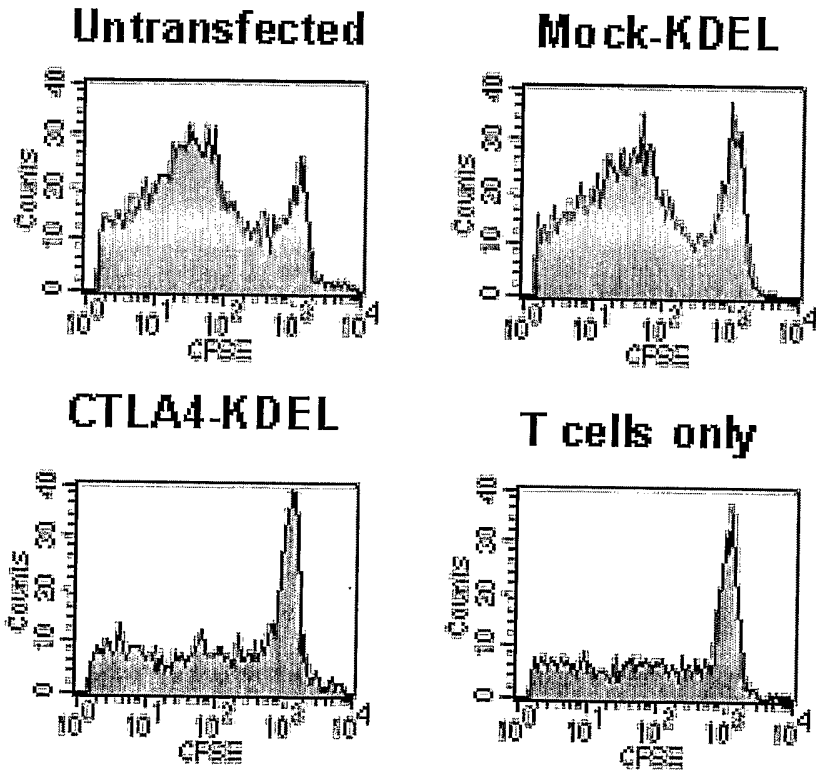
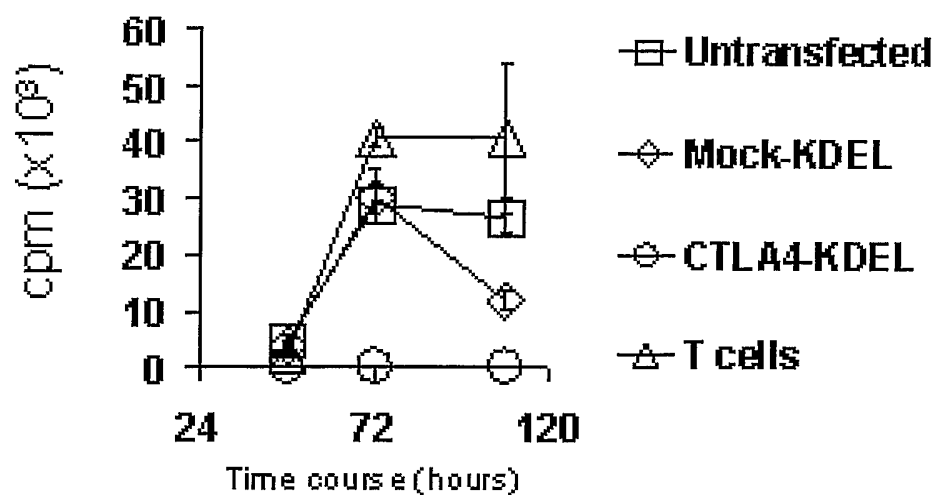


FIGURE 34



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FIGURE 35**FIGURE 36**