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(54) Title: IMMUNOMODULATORY POLYPEPTIDE

(57) Abstract: The invention relates to an isolated nucleic acid molecule and its corresponding polypeptide which has immunomodulatory activity in mammals and, in particular, provides protection against lymphocyte, and in particular NK cell, attack and, further, antibodies raised there against and antagonists active there against which also have immunomodulatory activity in relation to lymphocyte cell attack. The molecules of the invention are particularly suitable for treating viral disorders, inflammation, graft rejection, arthritis or autoimmune disease.



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IMMUNOMODULATORY POLYPEPTIDE

The invention relates to a novel, isolated nucleic acid and its corresponding polypeptide, which is of particular use as an immunomodulatory molecule, including means derivable therefrom or relating thereto such as vectors and primers. Moreover, the invention also relates to a method of immunomodulation using said nucleic acid or polypeptide in order to suppress the activity of Natural Killer (NK) cells of the immune system. Further, the invention relates to a pharmaceutical composition comprising either said nucleic acid or said polypeptide or an agent that blocks the activity of same.

The immune system is made up of a number of immune cells including lymphocytes. These cells are stem cell derived and differentiate into any one of a number of specialised cells which are adapted to perform a particular function within the complex cascade of events that characterise the immune system. Approximately 2% of lymphocytes circulating in the blood are neither T cells nor B cells but Natural Killer (NK) cells whose function is, amongst others, to respond to viral infection by recognising and bringing about the destruction of virus-infected cells. NK cells have receptors that transmit an inhibitory signal if they encounter class I MHC molecules on a cell surface. Certain viruses suppress MHC class I expression in cells and this can contribute towards rendering infected cells more susceptible to killing by NK cells. The same can also be true of cancer cells which have a reduced, or no, class I MHC expression and are therefore also more susceptible to killing by NK cells.

In addition to killing target cells, NK cells secrete cytokines such as anti-

viral cytokines and inflammatory cytokines. It therefore follows that NK cells are involved in inflammatory reaction(s) typically initiated as a result of the detection of foreign tissue, but also, inflammatory reactions triggered by non-foreign tissue such as in various autoimmune diseases.

5 During the course of evolution, to ensure their survival viruses have had to respond to effective host immune defences by evolving proteins that reduce the effectiveness of the host immune system. Thus, there exists a complex interplay between the immune system and the invading virus as the former embarks upon destruction of the latter and the latter retaliates by producing a series of proteins aimed at deceiving or side stepping the cells of the immune system.

10 Human cytomegalovirus (HCMV), a member of the herpesviridae, produces a number of gene products (e.g. gpUL40, gpUL18, gpUS6, gpUS2, gpUS3, gpUS11) that have been shown to modulate the nature of the immune response. After infection the virus establishes lifelong persistent infection in its host while successfully evading elimination by the immune response. Indeed, the virus has become a paradigm for virus immune evasion. HCMV encodes an impressive arsenal of genes that have been implicated in modulating the host immune response, including genes that downregulate expression of both MHC-I and MHC-II molecules that have a role in presenting antigen to T cells, an IL-10 homologue, a TNF receptor homologue, chemokines, chemokine receptors and IgG binding proteins.

20 HCMV has a worldwide distribution infecting up to 100% of the population in developing countries and more that 50% of the adult population in developed

countries. HCMV is capable of crossing the placenta and is the most common viral cause of congenital malformation in humans.

In most instances, the 'non-specific' symptoms of HCMV primary infection go unrecognised and are controlled by the host immune system. However, the virus is not eradicated with primary infection being accompanied by lifelong perisistence in its host.

However infection with this virus is of particular concern in immunocompromised individuals, especially those suffering from HIV. Other individuals at risk include all graft recipients, the elderly and the newborn, each of these groups may develop HMCV as a disease. Treatment can involve the use of viral polymerase inhibitors which aim to reduce viral proliferation in patients who develop the clinical symptoms associated with HMCV disease. The virus can be transmitted via saliva, urine, cervical and vaginal secretions, semen, breast milk, tears, faeces and blood. After primary infection, infectious viruses are excreted for prolonged periods which enhances the spread of HMCV.

Following infection, HMCV spreads locally to lymphoid tissues and then systemically in circulating lymphocytes and monocytes to the lymph nodes and spleen. The infection localises in the duct and epithelial cells of salivary glands, kidney tubules, cervix, testes and epididymis. However, pathologic changes are minor in a immunocompetent host and infection is generally asymptomatic. In other cases, infection leads to clinical disorders with potential complications including pneumonia, hepatitis, meningitis and autoantibody production.

In the immunocomprised host, such as an individual who has had a renal

transplant, HCMV can lead to renal dysfunction and graft rejection; in bone marrow/heart-lung transplant recipients HCMV can lead to severe respiratory problems, including pneumonia; in HIV patients HCMV can lead to gastrointestinal lesions, CNS disorders such as brain inflammation and damage to the retina and cochlea, causing blindness and deafness.

HCMV is difficult to treat. Acyclovir, which is commonly used to treat other herpes viruses is generally ineffective. Two other agents are therefore more generally used Ganciclovir and Foscarnet. These two agents have been shown to have strong *in vitro* activity against HCMV. However resistance to both agents is becoming increasingly common in the population especially amongst AIDS patients.

It therefore follows that due to the prevalence of this virus in the population its ability to reappear for long periods (indeed throughout an adult life), its ability to be reactivated at times of immunosuppression and the irreversible nature of some of its symptoms, it remains an important virus to treat.

The Natural Killer cell response is crucial in combating HCMV and our recent studies have focussed on this arm of the immune response.

We discovered our invention as a result of a chance observation. UL40 is an HCMV encoded gene that acts to upregulate cell surface expression of HLA-E, a non-classical MHC molecule encoded by the host cell. HLA-E binds to an inhibitory receptor expressed on approximately 50% NK cells to suppress NK functions; thus UL40 helps suppress NK killing of HCMV-infected cells [2,3]. While investigating the effect of UL40 on NK cells, we observed that HCMV

clinical isolates or the low passage Toledo strain provided significantly higher levels of protection against NK cell attack than the laboratory isolate strains AD169 or Towne (Figure 1). This led us to speculate that the Toledo strain may encode additional NK evasion functions not encoded by strains AD169 and Towne. Strain AD169 and Towne have been passaged extensively *in vitro* during which time a series of defects have accumulated in its genome, including a ~13 kb deletion in Towne and a ~15kb deletion in strain AD169 affecting the same region of the genome, designated ULb. We speculated that an NK inhibitory function could be encoded by a gene within this large deletion lost by strains AD169 and Towne, or alternatively, by a defective gene elsewhere on the genome. The recombinant virus HCMV Tx4 is based on strain Towne but has had the ULb sequence from strain Toledo has been artificially inserted. Significantly, the capacity of HCMV Tx4 to induce protection to NK cell-mediated cytotoxicity is much stronger than that observed with strain Towne and similar to that observed with strain Toledo (Fig 1C). Designated open reading frames (ORFs) from the 15kb element were cloned and were expressed in continuous cell lines as GFP fusion proteins. We discovered, during this process, that the published strain Toledo 15kb sequence contained a significant number of errors and that the prediction of ORF designations were not optimal. We therefore reanalysed this region of the HCMV genome.

During a reassessment of the strain Toledo ULb sequence, the observation was made that UL142 exhibited homology to HCMV UL18. gpUL18 is proposed to have a role in HCMV evasion of NK cells. Both UL141 and UL142 had previously been identified as having "MHC-like" folds. This

observation was, however, made with an erroneous version of UL141. A “re-defined” accurate version of UL141 is detailed in Figure 3 and is designated as IEF-1 to distinguish it. We cloned UL142 as a candidate NK evasion gene and IEF-1 (UL141b) (as a control construct) into the Adeasy-1 vector system. Unexpectedly, IEF-1 (UL141b) provided efficient protection against NK killing while UL142 exhibited no obvious effect in preliminary assays using NK lines (not shown). More detailed analysis confirmed UL141 expression induced NK protection whether continuously expressed in cell lines (Fig 2A) or introduced by means of an adenovirus vector (Fig 2B-C). Protection was elicited against established NK lines (NKL and SAM), transient unselected bulk NK cultures generated by interferon stimulation, primary NK lines and expanded NK clones (Table 2). By using both NK cell effectors and target cells from the same donor it provides an exact match of MHC molecules (an autologous assay).

IEF-1 was thereby unexpectedly identified as the novel HCMV-encoded NK evasion gene.

As previously mentioned, it is known that HCMV produces a number of gene products that modulate the cellular immune response. We describe herein a novel HCMV gene product that possess immunomodulatory activity. The gene product is effective at suppressing the activity of lymphocytes, and in particular, NK cells. The exact mechanism of this is actually unknown. It may act to actively suppress NK cell function, it may act to induce a factor that renders the viral target cell resistant to NK cell attack or some other mechanism. Whatever the means, the peptide we herein describe provides protection against attack by lymphocytes, and in particular, NK cells. It therefore follows that such an

immunomodulatory peptide is useful in regulating the immune system and in particular in the manufacture of antagonists, typically antibodies, that can suppress the function of this peptide and so facilitate the natural activity of lymphocytes, and in particular, NK cells in response to viral infection, particularly but not exclusively, HCMV infection. Further the immunomodulatory peptide is also useful in suppressing or circumventing the activity of lymphocytes, and in particular, NK cells in classical graft rejection or in their role in inflammation, or in their role in lymphocytosis disorders, or indeed any other disorder characterised by the participation of lymphocytes, and in particular, NK cells where it may be advantageous to suppress the effects of lymphocytes, and in particular, NK cells.

Summary of the Invention

According to a first aspect of the invention there is therefore provided an isolated nucleic molecule comprising the sequence structure shown in Figure 3 and designated HCMV IEF-1 or a nucleic acid molecule that is homologous thereto and is effective at providing protection against lymphocyte, and in particular NK, cell attack.

According to a further aspect of the invention there is therefore provided an isolated nucleic acid molecule comprising the sequence data shown in Figure 3 and designated HCMV IEF-1, or a nucleic acid molecule that is substantially homologous thereto, or a nucleic acid molecule that binds thereto under stringent hybridisation conditions.

According to a further aspect of the invention there is provided an isolated nucleic acid molecule comprising the sequence data shown in Figure 3 and

designated HCMV IEF-1, or a nucleic acid molecule that encodes a protein that is related to the HCMV IEF-1 protein as determined using a standard nucleic acid database search engine to search a nucleic acid database.

Homologues of IEF-1 are readily identified by searching standard
5 databases such as the GenBank database with a standard search engine such as BlastP, with default parameters. Ideally homologous molecules are identified as having a E value greater than $1e-5$.

In a preferred embodiment of the invention said nucleic acid molecule encodes an immunomodulatory molecule that is, most preferably, effective at
10 modulating the activity or effectiveness of NK cells and ideally a broad range of said cells including $CD94^+$ and $CD94^-$ cells. More preferably still, said nucleic acid molecule is effective at suppressing the activity of NK cells.

In a further preferred embodiment of the invention said nucleic acid molecule encodes a polypeptide that is effective at suppressing inflammation,
15 graft rejection, rheumatoid arthritis or autoimmune disorders.

According to a further aspect of the invention there is provided a vector comprising at least one of the aforementioned nucleic acid molecules. In a preferred embodiment of the invention said vector comprises multiple copies of said nucleic acid molecule and a suitable complement of control sequences for
20 effecting expression of said nucleic acid molecule.

According to a further aspect of the invention there is provided a cell comprising the aforementioned vector, ideally said cell is a mammalian, insect, bacteria or yeast cell.

According to a further aspect of the invention there is provided a method

for producing the polypeptide encoded by said nucleic acid molecule comprising culturing said transfected host cell and harvesting the intra or extracellular polypeptide encoded by said nucleic acid molecule. Most ideally, said host cell is transfected with a vector comprising said nucleic acid molecule and a
5 secretion signal whereby upon production of said polypeptide, said polypeptide is provided with a secretion signal and so cellular processing arranges for secretion of said polypeptide. Thereafter, said polypeptide can be harvested from the extracellular medium using conventional means.

According to a further aspect of the invention there is provided a nucleic
10 acid primer or probe comprising a nucleic acid molecule that is identical to or complementary to a part of the aforementioned nucleic acid molecule.

Ideally, said probe or primer is an oligonucleotide comprising at least six nucleotides.

According to yet a further aspect of the invention there is provided a
15 polypeptide encoded by said nucleic acid molecule. Said polypeptide comprises the sequence structure shown in Figure 3 and designated HCMV IEF-1, or a sequence structure substantially homologous thereto.

In a preferred embodiment of the invention said polypeptide possess immunomodulatory activity and is particularly characterized by its ability to
20 suppress the activity of NK cells. It therefore follows that the immunomodulatory polypeptide is effective in treating symptoms of the immune system characterised by inflammation, graft rejection, arthritis or autoimmune disease.

According to a further aspect of the invention there is provided an antibody to said polypeptide. Ideally, said antibody is monoclonal. Ideally, said

antibody, polyclonal or monoclonal, binds to HCMV IEF-1 and is thus useful in blocking the immunomodulatory activity of this viral gene product. It therefore follows that the antibody of the invention can be used to overcome the ability of a virus to evade the host immune response. Accordingly, the antibody of the invention is useful in the treatment of the viral infection and particularly HCMV viral infection.

According to yet a further aspect of the invention there is provided a soluble molecule comprising said polypeptide. More ideally, said soluble molecule is homologous to the sequence structure shown in Figure 5 and designated HCMV IEF-1 but without the underlined hydrophobic sequence.

The absence of this sequence facilitates the solubilisation of the polypeptide.

According to a yet further aspect of the invention there is provided an antagonist to said polypeptide. The antagonist is useful in blocking the immunomodulatory activity of the viral gene product. It therefore follows that the antagonist can be used to help overcome the ability of a virus to evade the host immune response. Accordingly, the antagonist of the invention is useful in the treatment of viral infection and particularly HCMV viral infection.

According to a further aspect of the invention there is provided a method of immunomodulation in a mammal which comprises administering to said mammal a therapeutically effective amount of immunomodulatory polypeptide wherein said immunomodulatory polypeptide has an immunomodulatory effect in said mammal.

Administering the immunomodulatory polypeptide of the invention, IEF-1,

suppresses the effectiveness of NK cells, thus their ability to respond to viral infection is suppressed but also, advantageously, so will be their ability to participate in an inflammatory response. It therefore follows that the immunomodulatory polypeptide of the invention can be used to suppress the activity of NK cells where there is an advantage to be achieved. Thus, most preferably said immunomodulation involves alleviating the symptoms of inflammation, graft rejection, arthritis or autoimmune disease.

According to a further aspect of the invention there is provided a pharmaceutical composition comprising said immunomodulatory polypeptide. Most preferably said composition comprises a therapeutically effective amount of said polypeptide, optionally, in conjunction with a pharmaceutically acceptable carrier. More preferably still said composition is formulated for the treatment of an immunomodulatory disorder such as inflammation, graft rejection, arthritis or autoimmune disease.

According to a further aspect of the invention there is provided a method of immunomodulation in a mammal which comprises administering to said mammal a therapeutically effective amount of antibody raised against said immunomodulatory polypeptide or a therapeutically effective amount of said antagonist wherein said antibody, or antagonist, has an immunomodulatory effect in said mammal.

Administering an antibody raised against said immunomodulatory polypeptide of the invention, IEF-1, results in the antibody binding to HCMV IEF-1 and thus blocking the activity of same. Accordingly, the antibody of the invention acts an immunomodulatory agent to overcome the ability of the virus to

evade the host immune response. It therefore follows that the antibody of the invention is useful when treating viral diseases.

Administering an antagonist of said immunomodulatory polypeptide of the invention, IEF-1, results in the antagonist blocking the activity of the immunomodulatory polypeptide. Accordingly, the antagonist of the invention acts as an immunomodulatory agent to overcome the ability of the virus to evade the host immune response. It therefore follows that the antagonist of the invention is useful when treating viral diseases.

According to a further aspect of the invention there is provided a pharmaceutical composition comprising said antibody and/or antagonist of the invention. More preferably said composition comprises a therapeutically effective amount of said antibody or antagonist, optionally, in conjunction with the pharmaceutically acceptable carrier. More preferably still said composition is formulated for the treatment of a viral disorder and, in particular, HCMV viral infection.

An embodiment of the invention will now be described by way of example only with reference to the following methods and figures wherein:

Figure 1 shows how infection of human fibroblasts with Toledo isolate of HCMV confers significantly stronger protection from NK killing than infection with AD169 laboratory strain. Toledo therefore appears to be using evasion strategies additional to and independent from the ones of AD169.

Figure 2 shows how expression of IEF-1 protects targets from NK lysis

Figure 3 shows the nucleic acid and amino acid sequence structure of IEF-1.

Figure 4 shows the amino acid alignment of IEF-1 in various clinical isolates of HCMV. A collection of clinical HCMV isolates was sequenced through IEF-1 coding region. IEF-1 sequence was present in all clinical isolates and was found to be highly conserved.

5 Figure 5 shows alignment of UL14 and IEF-1 for human and chimpanzee cytomegaloviruses and illustrates that they are members of the same gene family.

Figure 6 shows the immunomodulatory nature of IEF-1 (UL141) using strains (AD169, TB40, and Toledo) that differentially express IEF-1.

10 Figure 7 shows the targeting of a proportion of the IEF-1 protein to the cell surface.

Table 1 shows the results of experiments measuring NK lysis of HCMV infected cells.

15 Table 2 shows the results of experiments measuring NK lysis of cells expressing IEF-1 (UL141).

Methods

Cells

20 Target cells used in experiments were human primary fibroblasts, **HFFF**, **DD-SF**, **D3SF**, **D7SF** grown in DMEM (Life Technologies); supplemented with 10% foetal calf serum (Life Technologies) and antibiotics (Life Technologies).

Effector cells used in experiments were as follows. NK clone **NKL**¹; NK line **DD-NK3**²; NK lines **SAM** and **SAM-8** were derived as described previously, from the same patient as NK line **DEL**² and displayed the same phenotype as DEL. Primary NK lines, D3NK and D7NK, were derived from lab donors (D3 and

D7) by stimulation of PBMC with IL-2 and regular feeds with irradiated, allogenic PBMC. The lines were regularly depleted of CD3⁺T cells before use in assays. Primary polyclonal **PBMC** were isolated from buffy coats (University Hospital of Wales bloodbank) on Histopaque gradient (Sigma) and depleted of adherent
5 cells. The cells were then stimulated overnight with 500U/ml of interferon- γ and used for an assay. All NK cells were grown in RPMI (Life Technologies), 10% human heat inactivated AB serum (University Hospital of Wales bloodbank) and 1,000 U/ml of recombinant human IL-2 (Proleukin, Chiron),

293 or **911** cells were used for adenovirus and HFFF were used for
10 HCMV growth and titrations. **A549** cells were used for production of soluble IEF-1. All were maintained as described above.

Viruses and Infections

HCMV strains and recombinants used were as follows. Laboratory strain **AD169** was provided by Dr J Booth (St Georges Hospital); the AD169 UL40
15 deletion mutant **AD169 Δ UL40** has been described previously²; the low passage clinical isolate **Toledo**, the laboratory strain **Towne** and Towne/Toledo recombinant HCMV **Tx4** was provided by Dr E Mocarski (Stanford University, USA); clinical isolate **Merlin** was provided by Dr L Neale (University of Wales), strain **Duff** was derived from clinical isolate 3157 by 3 rounds of plaque
20 purification; isolate 3157 was provided by Dr J Fox (University of Wales). Duff strain was selected because of an interesting mutation, found within UL40 ORF. The start ATG codon (M) of UL40 in 3157 isolate was found to be mutated to ACG (T) (not published) and because the next available ATG codon is 45 nucleotides downstream of the original UL40 start site, the putative isolate 3157

UL40 protein would not have the HLA-E binding peptide ³. Neither isolate 3157 or strain Duff were fully characterised, except of the UL40 ORF. Clinical isolate **TB40** was provided by Christian Sinzger (Germany), strains **Bart** and **Lisa** were derived from this isolate by plaque purification. Bart has a mutation leading to a frameshift in UL141 ORF, and thus no UL141 protein expression. HFFF or DD-SF were infected with 10 PFU/cell of the relevant HCMV at 37°C on a rocker for 2 hours. Cells were then rinsed and incubated for 72 hours prior to chromium release assay ².

Construction of replication deficient adenoviruses, RAdS, was described previously ⁴ and was based on AdEasy-1 vector system containing a deletion of the E1 gene region, with the transgene inserted downstream of the HCMV MIE promoter ⁴. **RAd502** was generated as follows. Plasmids pAdTrack-CMV and pAdEasy-1 (both provided by Dr B Vogelstein (John Hopkins Oncology Centre, USA)) were re-combined in *E.coli* BJ5183, generating pAL502. Purified pAL502 DNA was then transfected into 911 cells, generating viable virus RAd502. RAd502 was used as a control virus throughout the study. RAd502 drives the expression of GFP in infected cells. **RAd592** was generated as follows. Plasmids pShuttle-CMV and pAdEasy-1 (both provided by Dr B Vogelstein (John Hopkins Oncology Centre, USA)) were re-combined in *E. coli* BJ5183, generating pAL592. Purified pAL592 DNA was then transfected into 911 cells, generating viable virus RAd592. RAd592 was used as a control virus throughout the study. RAd592 does not drive the expression of any transgene. **RAd522** was generated as follows. IEF-1 gene was amplified from Toledo genomic DNA by PCR using AGS Gold polymerase (Hybaid) with the following

primers on PCR-Express Hybaid thermal cycler. T108 = 5'-ATCATGTGCCGCCGGGAGTCG-3', 130 = 5'TCACCTCTTCATCTTTCTAAC-3' (Life Technologies). The IEF-1 PCR product was inserted into pCR2.1-TOPO vector (Invitrogen), generating pAL510. The sequence of the inserted IEF-1 was then checked by sequencing, using BigDye Terminator cycle sequencing kit (Applied Biosystems) on ABI PRISM 377 sequencer. The IEF-1 was cut from pAL510 as a *NotI/HindIII* fragment and inserted into *NotI/HindIII* cut pAdTrack-CMV, generating pAL516. The pAL516 was re-combined with pAdEasy-1 in *E. coli* BJ5183, generating pAL522. Purified pAL522 DNA was then transfected into 911 cells, generating viable virus RAd522. RAd522 drives the expression of GFP and IEF-1 in infected cells. **RAd587** was generated as follows. IEF-1 gene was cut from pAL510 as *NotI/HindIII* fragment and ligated into *NotI/HindIII* cut pShuttle-CMV, generating pAL580. The pAL580 was re-combined with pAdEasy-1 in *E. coli* BJ5183, generating pAL587. Purified pAL587 DNA was then transfected into 911 cells, generating viable virus RAd587. RAd587 drives the expression of IEF-1 in infected cells. **RAd550** was generated as follows. IEF-1 gene (lacking transmembrane and cytoplasmic regions, which were placed with streptag (IBA, Germany)) was amplified from pAL510 by PCR using AGS Gold polymerase (Hybaid) with the following primers on PCR-Express Hybaid thermal cycler. T139 = 5'-GGGGTACC ATCATGTGCCGCCGGGAGTCG-3', 140 = 5'-CCGCTCGAGTTATTTTTCGAACTGCGGGTGGCTCCAAGCGCTCCGAGTGG CCCAGGGAGACATC-3' (Life Technologies). The IEF-1 PCR product was digested with *KpnI* and *XhoI* and inserted into *KpnI/XhoI* cut pAdTrack-CMV

vector, generating pAL542. The pAL542 was re-combined with pAd-Easy-1 in *E. coli* BJ5183, generating pAL550. Purified pAL550 DNA was then transfected into 911 cells, generating viable virus RAd550. RAd550 drives the expression of GFP and soluble IEF-1 in infected cells. The soluble IEF-1, designated P-550, was prepared as follows. A549 cells were infected with RAd550 and maintained in serum free medium for 4 days. The medium was harvested and soluble IEF-1 was purified on Streptactin Macroprep column (IBA, Germany) according to manufacturer's instructions. The identity of purified protein was verified by western blot with anti-streptag monoclonal antibody (IBA) and by direct N-terminal protein sequencing on Applied Biosystems Procise Sequencer (LSUMC Core Laboratories, USA).

HFFF were infected with 200 PFU/cell of the relevant adenovirus recombinant at 37°C on a rocker for 2 hours. DD-SF were infected with 1000 PFU/cell of the relevant RAd at 37°C on a rocker for 2 hours. Cells were then rinsed and incubated for 72 hours prior to chromium release assay ².

Antibodies and miscellaneous methods

P-550 was used to raise polyclonal mouse antibody M-550 (Moravian Biotek, Czech Republic). This antibody was used on western blots to confirm the expression of IEF-1 in infected cells. The cells were infected and treated as described above and then extracted in NP-40 lysis buffer (150mM NaCl, 1% NP-40, 50mM Tris pH 8, protease inhibitors cocktail (Sigma) and digested with Endoglycosidase H (EndoH) or Peptide-N-glycosidase F (PNGaseF) (NEB), according to manufacturer's instructions.

Infection of Human Fibroblasts with Toledo Isolate of HCMV

Infection of human fibroblasts with HCMV strain Toledo confers significantly stronger protection from NK killing than infection with AD169 laboratory strain (Fig 1). Toledo therefore appears to be using evasion strategies addition to and independent from the ones of AD169.

5 Human foreskin foetal fibroblasts (HFFF) were infected for 72 hours with 10 PFU/cell of the following strains and recombinants of human cytomegalovirus (HCMV); **AD169** and **Towne** strains. HCMV suffers from genetic instability, consequently not all versions of strains AD169 and Towne in use worldwide are identical. The version of strain AD169, originally provided by Dr J. Booth, was
10 from the same stock used to generate the clones sequenced at the University of Cambridge and is known to lack approximately 15kb of sequence present in clinical isolates. The stock of strain Towne, supplied by Prof E.S. Mocarski (Stanford University, California), is known to contain a deletion of approximately 13kb. These deletions are assumed to have occurred during laboratory passage.
15 The approximately 15kb of sequence missing from strain AD169 is predicted to encode at least 23 unique ORFs designated UL128 through to UL150. The Towne genome has a similar deletion except it retains the UL146-148 ORFs (new Davison designation 2003) not present in AD169). Most of these ORFs are present in the genomes of low passage clinical isolate **Toledo** and other
20 clinical isolates of HCMV. HCMV **Tx4** is a recombinant virus where the lesion in Towne has been repaired with sequences from strain Toledo. HCMV Δ **UL40** is based on strain AD169 but the UL40 ORF has been disrupted by insertion of the gene for Green Fluorescent Protein.

Infected or mock infected cells were subjected to a standard Natural Killer

(NK) chromium release assay to establish the level of specific lysis for each of the targets. NK clone **NKL** (Fig. 1A, 1C) and NK line SAM (Fig. 1B) were used for these assays. Mock infected targets were used as controls to set-up and establish a baseline of killing for each effector. Infection with AD169 strain conferred a significant protection from lysis when compared to mock infected cells.

Infection of targets with AD169 Δ UL40 recombinant greatly induced killing, providing an evidence that the deleted UL40 gene product was responsible for NK modulation in this particular assay (Fig. 1A). Interestingly, infection of targets with Toledo isolate consistently offered significantly higher levels of protection than AD169 strain. Furthermore, infection of human fibroblasts with a series of low-passage HCMV clinical isolates also conferred substantially higher levels of protection against NK cell attack than did strain AD169 (not shown). Also, infection of targets with the HCMV clinical isolate Duff (containing a mutation within UL40 ORF and thus making Duff UL40 product non-functional), still resulted in significant protection from NK lysis, when compared to mock infected cells (not shown). Since this loss of UL40 function was not sufficient to render HCMV isolate Duff-infected cells sensitive to NK cell attack, it served as an additional evidence to support the hypothesis that NK inhibitory functions may be encoded by HCMV clinical isolates and strain Toledo which are not present in AD169 genome.

This hypothesis was further supported by a comparison of Towne strain and the Tx4 recombinant. Human fibroblasts infected with strains Towne and AD169 were susceptible to NK lysis at a similar level (Fig. 1C) but importantly cells

infected with HCMVTx4 (with the repair to the 13kbTowne deletion) was extremely resistant to NK lysis (Fig. 1C). This observation strongly suggested that by repairing the Towne lesion, wild type phenotype was also repaired, suggesting that at least one NK evasion function can be expected to map to the
5 UL128-UL150 region.

Table 1 shows similar results to the ones presented in Figures 1A and 1B but includes results performed with tissue type matched (autologous) effectors and targets.

Expression of IEF-1 and its Affect on NK Lysis

10 Expression and screening of a number of Toledo unique ORFs for their potential to modulate NK function identified one of the newly re-defined (details in Fig. 3) Toledo ORF, designated Immune Evasion Function-1 (IEF-1), as able to confer cells resistant to NK lysis (not shown).

After this initial screen of Toledo GFP fused ORFs using stably
15 transfected 293 cells as targets and NKL cells as effectors (partially presented in Fig. 1), the IEF-1 gene was expressed using a recombinant adenovirus vector. Human foreskin foetal fibroblasts (HFFF) were infected for 72 hours with 200 PFU/cell of the following adenovirus recombinants; RAd502 is an AdEasy1 recombinant expressing **GFP**, RAd522 is an AdEasy1 recombinant expressing
20 GFP and **IEF-1**. Targets infected with adenovirus expressing GFP were used to set-up and establish a baseline of killing. Expression of IEF-1 ORF conferred a significant protection from lysis, compared to control targets expressing GFP only, providing evidence that IEF-1 gene product could induce significant NK protection.

The following effectors were used to confirm this observation; pooled PBMCs stimulated overnight with 500 units of interferon- γ (Fig. 2B), NK clone NKL (Fig. 2C), NK line SAM (Table 2) and various primary NK lines matched or mismatched with the targets as indicated (Table 2).

5 UL141 ORF Re-Definition (correction of the database frame shift and incorrect ORF prediction)

Analysis of the Toledo genome sequenced in our laboratories revealed an important single base difference between our sequence and the Genbank submission. This observation was then confirmed in several clinical isolates of HCMV (not shown). Therefore, the original prediction of UL141 ORF (bases 5098-6376) had to be re-defined. Putative novel ORFs were identified (bases 5098-5414) and IEF-1 (bases 5359-6376). Since UL141 and UL142 ORFs have already been used in publications, the new ORF associated with NK evasion is designated IEF-1 in this submission to avoid confusion.

15 Characterisation of the IEF-1 Protein

A collection of clinical HCMV isolates (Fig. 4a, indicated on the left) was sequenced through IEF-1 coding region. IEF-1 sequence was present in all clinical isolates that were available at the time and the amino acid alignment showed IEF-1 to be highly conserved.

20 IEF-1 is predicted to be a glycoprotein containing potential transmembrane (box a), ER retrieval (box b), 3 glycosylation (boxes c) and nuclear targeting (box d) domains (Fig. 4a). To test the signal peptide prediction (box e), we have sequenced the N-terminus of affinity purified soluble IEF-1 (sIEF-1) secreted from human cells and presumably fully cleaved (Fig. 4b). The

sIEF-1 sequence revealed that the signal peptide was cleaved after the alanine at amino acid residue 36 (not shown). The molecular weight of sIEF-1 polypeptide chain was then calculated from its coding DNA sequence to be approximately 28kDa. The sIEF-1 migrated on denaturing SDS gel with an
5 apparent weight of >35kDa (Fig. 4b), which suggested further processing, like for instance glycosylation increasing the apparent molecular weight of the protein. Soluble IEF-1 was used to generate mouse polyclonal antibody M550.1 used in western blots and immunoprecipitations that follow.

The expression of IEF-1 could be detected by western blot in extracts of
10 cells infected with HCMV clinical isolates Toledo and Merlin, but not in the laboratory strain AD169 known to have a natural IEF-1 deletion (Fig. 4c). Interestingly, the IEF-1 detected in HCMV extracts was susceptible to either EndoH or PNGaseF endoglycosidase digestion. This confirmed that IEF-1 was a glycoprotein and the sensitivity to EndoH suggested immature glycosylation
15 pattern characteristic to intracellular, ER resident proteins. Expression of IEF-1 was also confirmed in cells infected with adenovirus recombinant encoding the IEF-1 gene, but not control adenovirus (Fig. 4d). In a western transfer of proteins harvested from a time course of HCMV-infected cells, IEF-1 was found to be expressed during both the early and late phases of infection; the IEF-1 gene
20 product could be detected by 2 days post infection, and progressively accumulates in HCMV-infected cells from then on (Fig. 4e).

To this point, we were unable to detect IEF-1 expression on the cell surface by FACS, using the available reagents. The IEF-1 expressed from the adenovirus vector was however readily detected and localised using

immunofluorescent intracellular staining (Fig. 4f). All these presented observations suggest that IEF-1 is an intracellular protein. However cell surface targeting can not be excluded until more sensitive reagents are developed and used.

5 Alignment of UL14 and UL141 (IEF-1) for Human and Chimpanzee Cytomegaloviruses

The alignment data is shown in Figure 5 where it can be seen that the high degree of homology indicates that the proteins are members of the same gene family. The underlined sequence shows the hydrophobic domain of the protein and it therefore follows that omission of this sequence will produce a soluble protein which may be favoured when producing pharmaceutical compositions.

10 Investigations using purified sequence variants of HCMV strain TB40

During routine screening of HCMV strains being used or newly introduced to the laboratory, we have observed that the expression levels of IEF-1 after infection with strain TB40 isolate were considerably lower than with other isolates (Fig. 6a). Sequencing of the IEF-1 gene revealed the existence of a mutation within the IEF-1 gene leading to a frameshift that would prevent expression of the IEF-1 protein. Interestingly, the level of protection from NK lysis achieved by infection with HCMV strain TB40 was midway between between levels observed following infection with strains AD169 (IEF-1⁻) and Toledo (IEF-1⁺) (Fig. 6b). We hypothesised that HCMV TB40 may be a mixed population of viruses encoding both intact and mutant forms of IEF-1. From the HCMV TB40 stock, we plaque purified two variants on the basis of IEF-1

expression: Bart (IEF-1⁻) and Lisa (IEF-1⁺) (Fig. 6c). When used in NK assays, we could now see a considerable split between these two strains with reference to their ability to protect the infected targets from killing, with the virus encoding IEF-1 (Lisa) bestowing enhanced levels of protection (Fig. 6d). This data implies that the expression of IEF-1 plays a crucial role NK evasion in the context of lytic HCMV infection.

Cell surface targeting of IEF-1

Some experiments are shown in Figure 7 that have been performed to establish whether IEF-1 was targeted to the cell surface. Cells infected with either HCMV TB40 variants Bart or Lisa were cell surface biotinylated, lysed and immunoprecipitated with the antibody M550.1 (Fig. 7a), or streptactin beads (Figs. 7b and 7c). The immunoprecipitated proteins were then analysed by western blot with streptactin-HRP (Fig. 7a) or M550.1 (Figs. 7b and 7c). Figure 7a shows no apparent cell surface IEF-1, while IEF-1 could be detected in biotinylated cell extracts. On the contrary Figure 7b suggests the presence of IEF-1 on the cell surface. We feel it is too early to conclude whether IEF-1 is targeted to the cell surface, but from the preliminary data presented here, one could infer the following. The precipitation method with streptactin beads is more sensitive than the method using M550.1 antibody, therefore in the case of only very small proportion of IEF-1 being targeted to the cell surface, it is likely to see such discrepancy. Comparing the amounts of IEF-1 in total cell extracts with the amounts of IEF-1 surface biotinylated and immunoprecipitated (not shown) it is safe to say that should IEF-1 be present on the cell surface, it may only be a very small proportion.

Although the biotinylation was performed quickly, at 4°C and in the presence of sodium azide, one cannot exclude the possibility that a proportion of the biotin label was internalised.

5 In conclusion, the data presented herein shows that we have isolated a novel immunomodulatory protein from HCMV. To avoid confusion with the prior art we have termed this protein IEF-1 and shown using *in vitro* experimentation that IEF-1 modulates the activity of NK cells by reducing the NK specific lysis.

References

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2. Wang, E.C.Y. *et al.* (2002) PNAS 99:7570-7575.
3. Tomasec, P. *et al.* (2000) Science 287:1031-1033
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Table 1 NK Lysis of HCMV Infected Cells, see also Figure 1

NK Line	Targets	NKs:Targets	Mock	+ AD169	+ Toledo
D3NK	D3SF	20:1	40±6	21±1	1±1
D7NK	D7SF	18:1	20±2	18±3	1±4
D3NK(Δ CD94)	D3SF	40:1	29±3	33±2	1±1
D7NK(Δ CD94)	D7SF	22:1	22±4	25±2	1±3

5

Table 2. NK lysis of cells expressing UL141, see also Figure 2

NK Line	Targets	NKs: Targets	GFP (control)	UL141
D3NK	D3SF*	20:1	30±2	13±5
D3NK	D3SF*	12:1	17±5	11±1
D3NK	HFFF	12:1	22±8	8±2
D7NK	D7SF*	18:1	20±2	8±2
D3NK(Δ CD94)	D3SF*	40:1	31±3	11±2
D7NK(Δ CD94)	D7SF*	22:1	16±4	7±3
D7NKp3.2	D7SF*	40:1	18±4	3±3
D7NKp3.2	HFFF	40:1	20±3	8±2
SAM	HFFF	40:1	40±2	19±9
SAM	HFFF	40:1	12±1	1±8
SAM	D7SF	40:1	20±2	7±3

autologous targets are indicated with *

CLAIMS

1. An isolated nucleic acid molecule comprising the nucleic acid sequence shown in Figure 3 and designated HCMV IEF-1, or a homologous molecule that provides protection against lymphocyte cell attack.
- 5 2. An isolated nucleic acid molecule according to Claim 1 comprising a nucleic acid sequence that is substantially homologous to IEF-1 or a nucleic acid molecule that binds thereto under stringent hybridisation conditions.
3. An isolated nucleic acid molecule comprising the sequence data shown in Figure 3 and designated HCMV IEF-1, or a nucleic acid molecule that encodes a
10 protein that is related to the HCMV IEF-1 protein as determined by a search of a nucleic acid database using a conventional search engine.
4. An isolated nucleic acid molecule according to claim 3 wherein said search engine is BlastP.
5. An isolated nucleic acid molecule according to any preceding claim that is
15 effective at suppressing the activity of NK cells.
6. An isolated nucleic acid molecule according to any preceding claim which is effective at modulating the activity of CD94⁺ and CD94⁻ NK cells.
7. An isolated nucleic acid molecule according to any preceding claim which is effective at suppressing any one or more of the following: inflammation,
20 graft rejection, rheumatoid arthritis or autoimmune disorder.
8. A vector comprising a nucleic acid molecule according to any preceding claim.
9. A vector according to claim 8 comprising multiple copies of said nucleic acid molecule.

10. A vector according to claim 8 or 9 which further comprises control sequences for the effective expression of said nucleic acid molecule(s).

11. A cell transformed or transfected with the vector of claims 8-10.

12. A cell according to claim 11 which is a mammalian, insect, bacteria or a yeast cell.

13. A method for producing a polypeptide encoded by the nucleic acid molecule of claims 1-7 comprising:

(i) culturing a transformed or transfected host cell according to claims 11 or 12 under conditions that are conducive to the expression of said polypeptide; and

(ii) harvesting the intra- or extra-cellular polypeptide encoded by said nucleic acid molecule.

14. A method according to claim 13 wherein said host cell comprises a nucleic acid molecule that is further provided with a secretion signal whereby, upon production of said polypeptide, said polypeptide undergoes cellular processing resulting in the secretion of same.

15. A method according to claim 14 wherein said polypeptide is harvested by extracting same from the extra-cellular medium.

16. A polynucleotide that is identical to, or complementary to, a part of a nucleic acid molecule according to claims 1-7.

17. A polynucleotide according to claim 16 wherein said polynucleotide comprises at least 6 nucleotides.

18. A polypeptide encoded by the nucleic acid molecule of claims 1-7.

19. A polypeptide according to claim 18 which comprises the amino acid

sequence structure shown in Figure 3 and designated HCMV IEF-1, or a sequence homologous thereto.

20. A polypeptide according to claims 18 or 19 wherein said polypeptide possesses immunomodulatory activity.

5 21. A polypeptide according to claim 20 wherein said polypeptide suppresses the activity of lymphocyte cells.

22. A polypeptide according to claim 21 wherein said polypeptide suppresses the activity of NK cells.

10 23. A polypeptide according to claims 20, 21 or 22 wherein said polypeptide is effective at modulating the activity of CD94⁺ and CD94⁻ NK cells.

24. A polypeptide according to claims 18-23 wherein said polypeptide is effective at suppressing one or more of the following: inflammation, graft rejection, arthritis or autoimmune disease.

25. An antibody raised against the polypeptide of claims 18-24.

15 26. An antibody according to claim 25 wherein said antibody is monoclonal.

27. The use of an antibody according to claims 25 or 26 in the treatment of viral infection.

28. The use of an antibody according to claim 27 wherein said infection is caused by HCMV.

20 29. A soluble polypeptide comprising the polypeptide according to claims 18-24 wherein the hydrophobic domain thereof has been excised or deleted.

30. A soluble polypeptide according to claim 29 comprising the amino acid sequence structure shown in Figure 5 minus the underlined hydrophobic domain.

31. A method of immunomodulation in a mammal which comprises administering to said mammal a therapeutically effective amount of immunomodulatory polypeptide according to claims 18-24 or 29-30 wherein said immunomodulatory polypeptide has an immunomodulatory effect in said mammal.

32. A pharmaceutical composition comprising the immunomodulatory polypeptide of claims 18-24 or 29-30.

33. A pharmaceutical composition according to claim 32 comprising a therapeutically effective amount of said polypeptide, in conjunction with a pharmaceutically acceptable carrier.

34. A pharmaceutical composition according to claims 32 or 33 wherein said composition is formulated for the treatment of any one or more of the following disorders: inflammation, graft rejection, arthritis or autoimmune disease.

35. A method of immunomodulation in a mammal which comprises administering to said mammal a therapeutically effective amount of the antibody according to claims 25 or 26 wherein said antibody has an immunomodulatory effect in said mammal.

36. A pharmaceutical composition comprising the antibody of claims 25 or 26.

37. A pharmaceutical composition according to claim 36 comprising a therapeutically effective amount of said antibody in conjunction with a pharmaceutically acceptable carrier.

38. A pharmaceutical composition according to claims 36 or 37 wherein said composition is formulated for the treatment of a viral infection.

39. A pharmaceutical composition according to claim 38 wherein said viral

infection is caused by HCMV.

40. A method of immunomodulation in a mammal which comprises administering to said mammal a therapeutically effective amount of an antagonist to the polypeptide of claims 18-24 or 29-30 wherein said antagonist
5 has an immunomodulatory effect in said mammal.

41. A pharmaceutical composition comprising the antagonist of claim 40.

42. A pharmaceutical composition according to claim 41 comprising a therapeutically effective amount of said antagonist in conjunction with a pharmaceutically acceptable carrier.

10 43. A pharmaceutical composition according to claims 41 or 42 wherein said composition is formulated for the treatment of a viral infection.

44. A pharmaceutical composition according to claim 43 wherein said infection is caused by HCMV.

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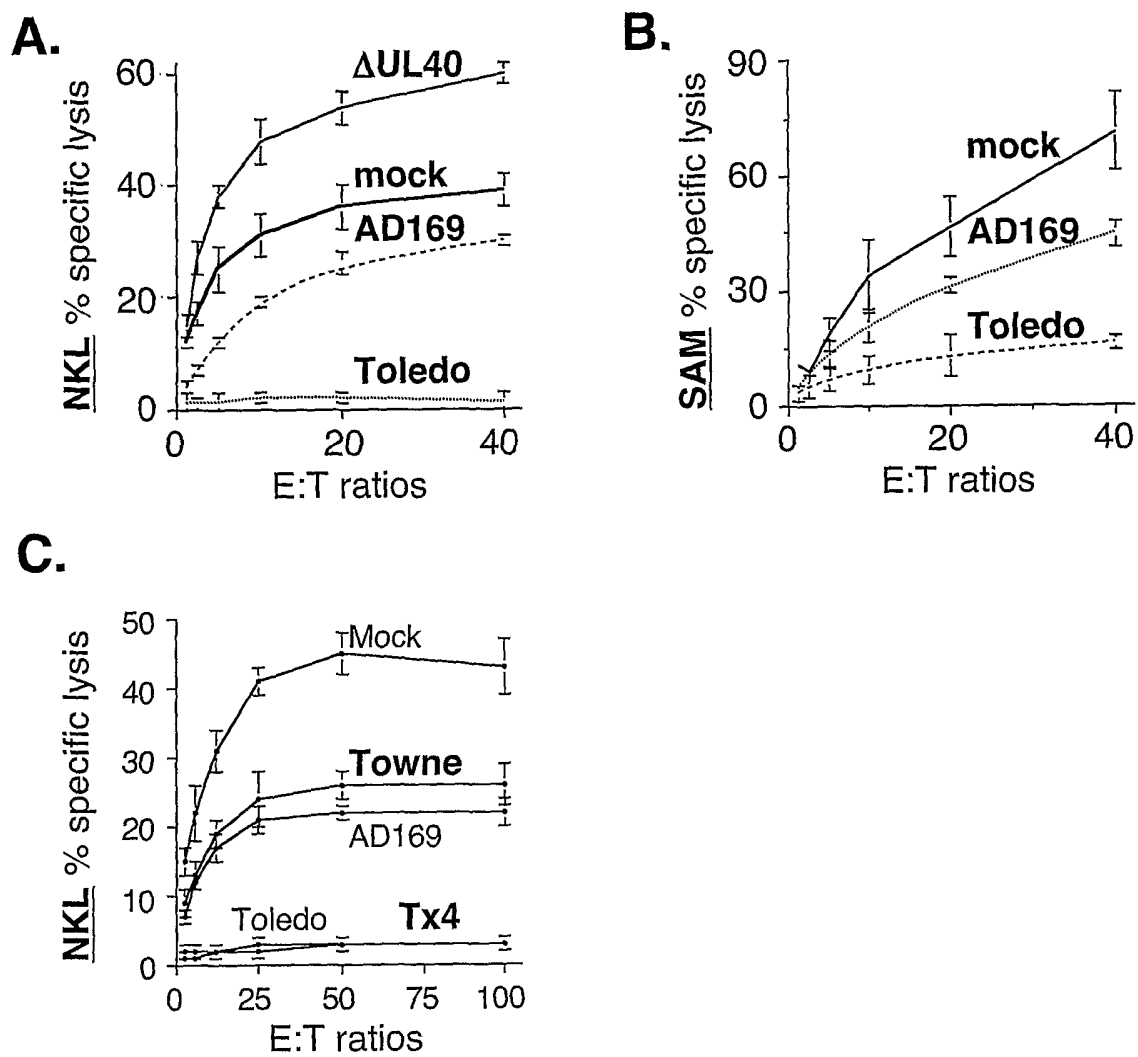


FIGURE 1

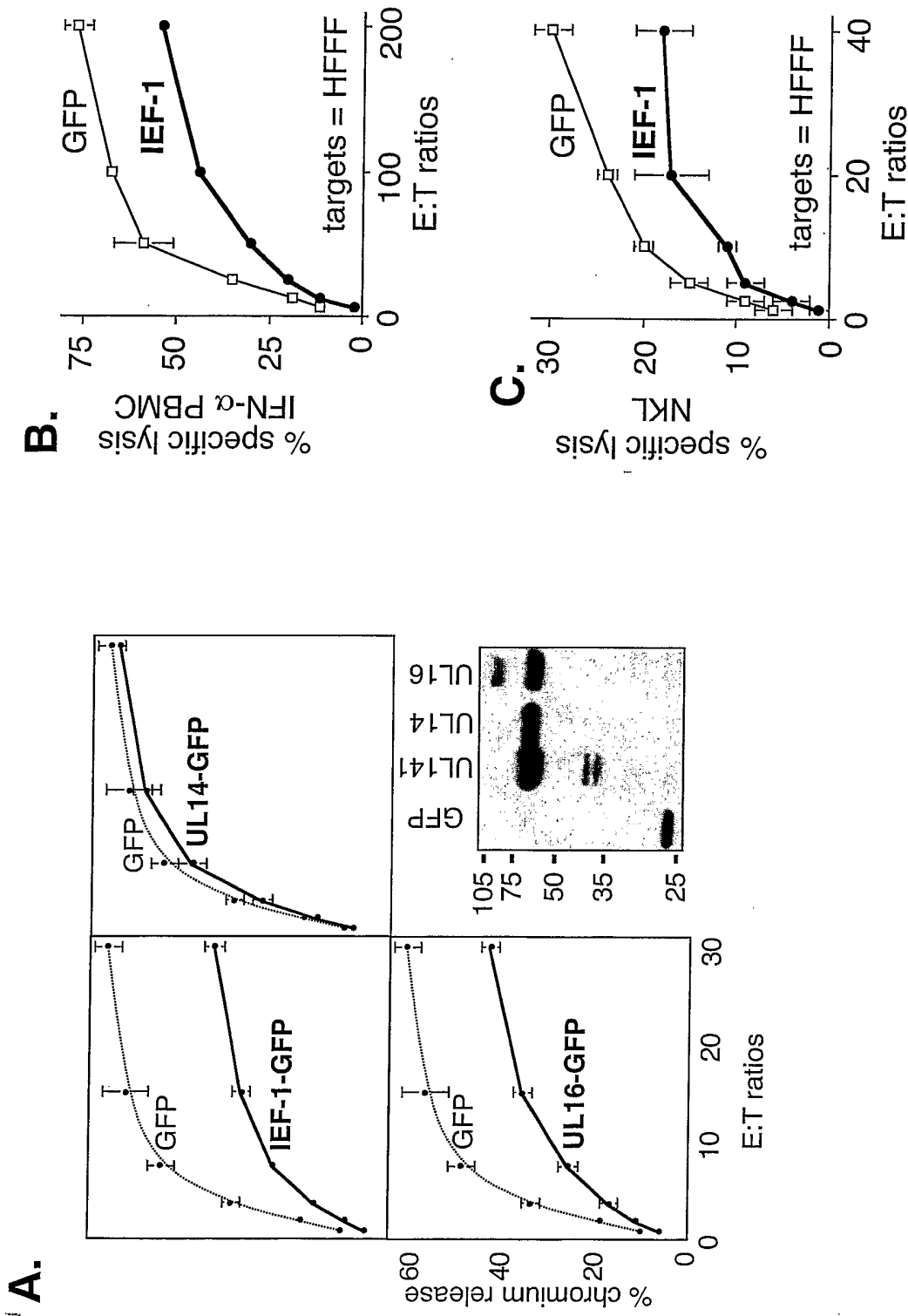


FIGURE 2

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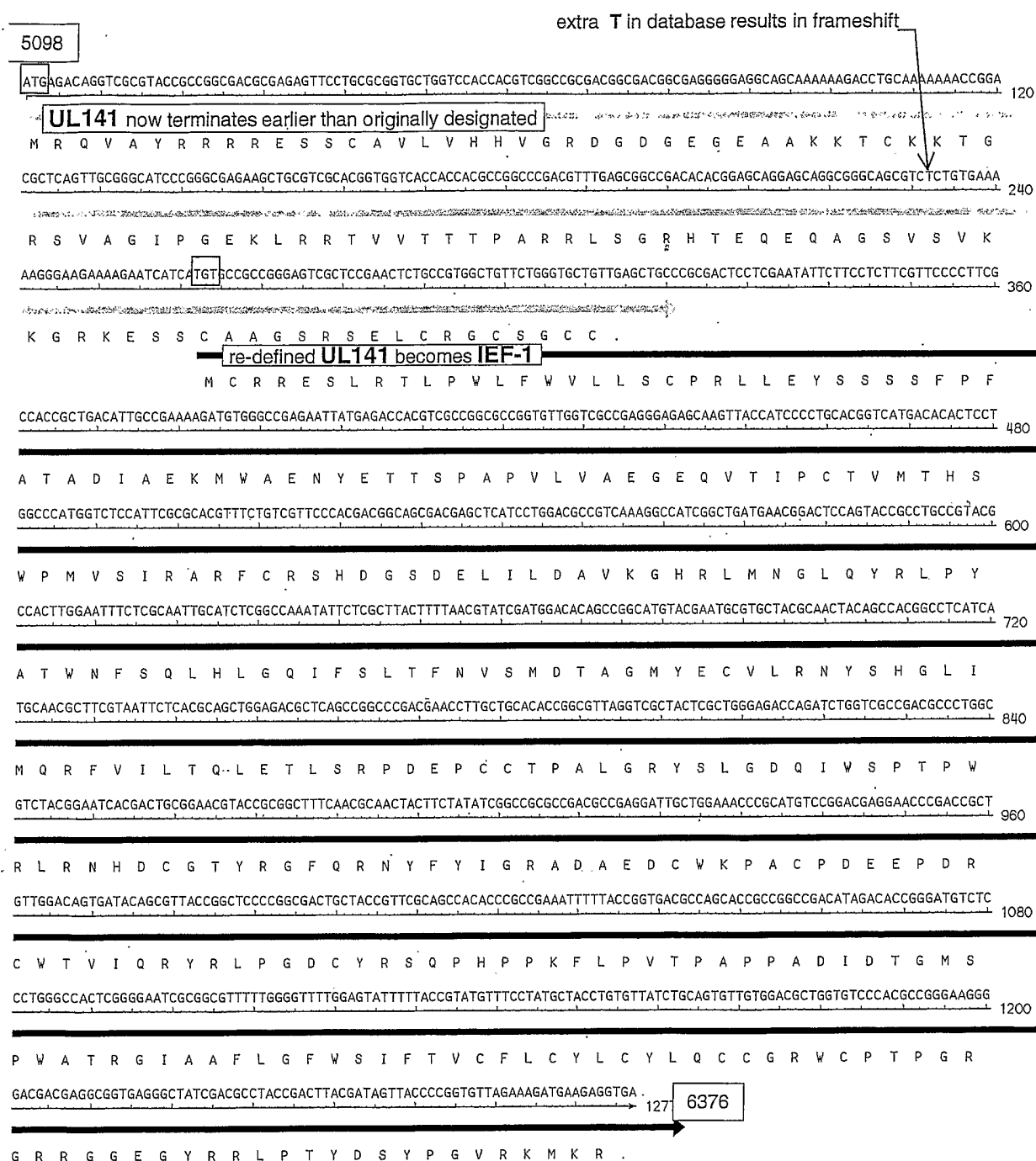


FIGURE 3

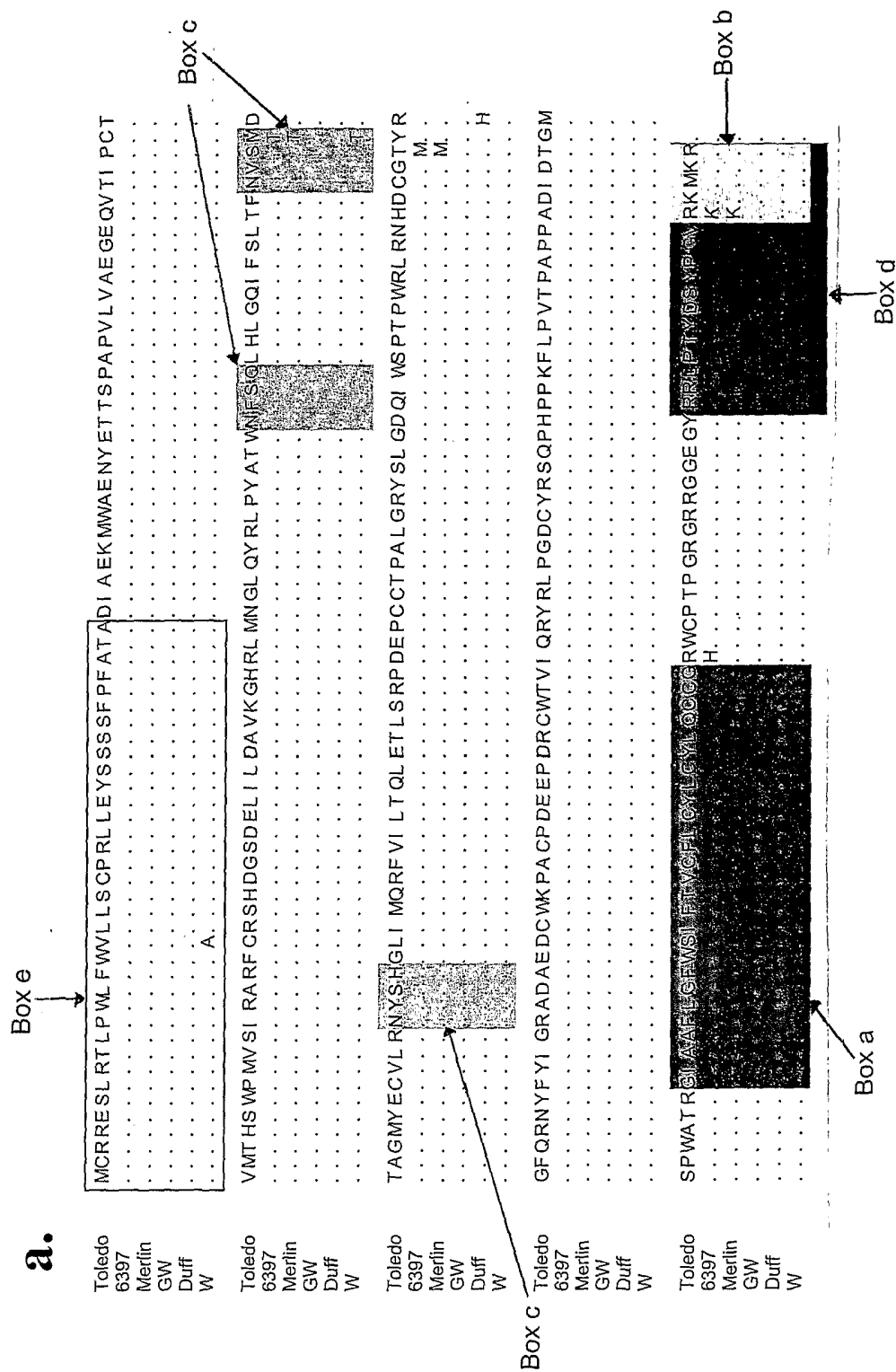


FIGURE 4

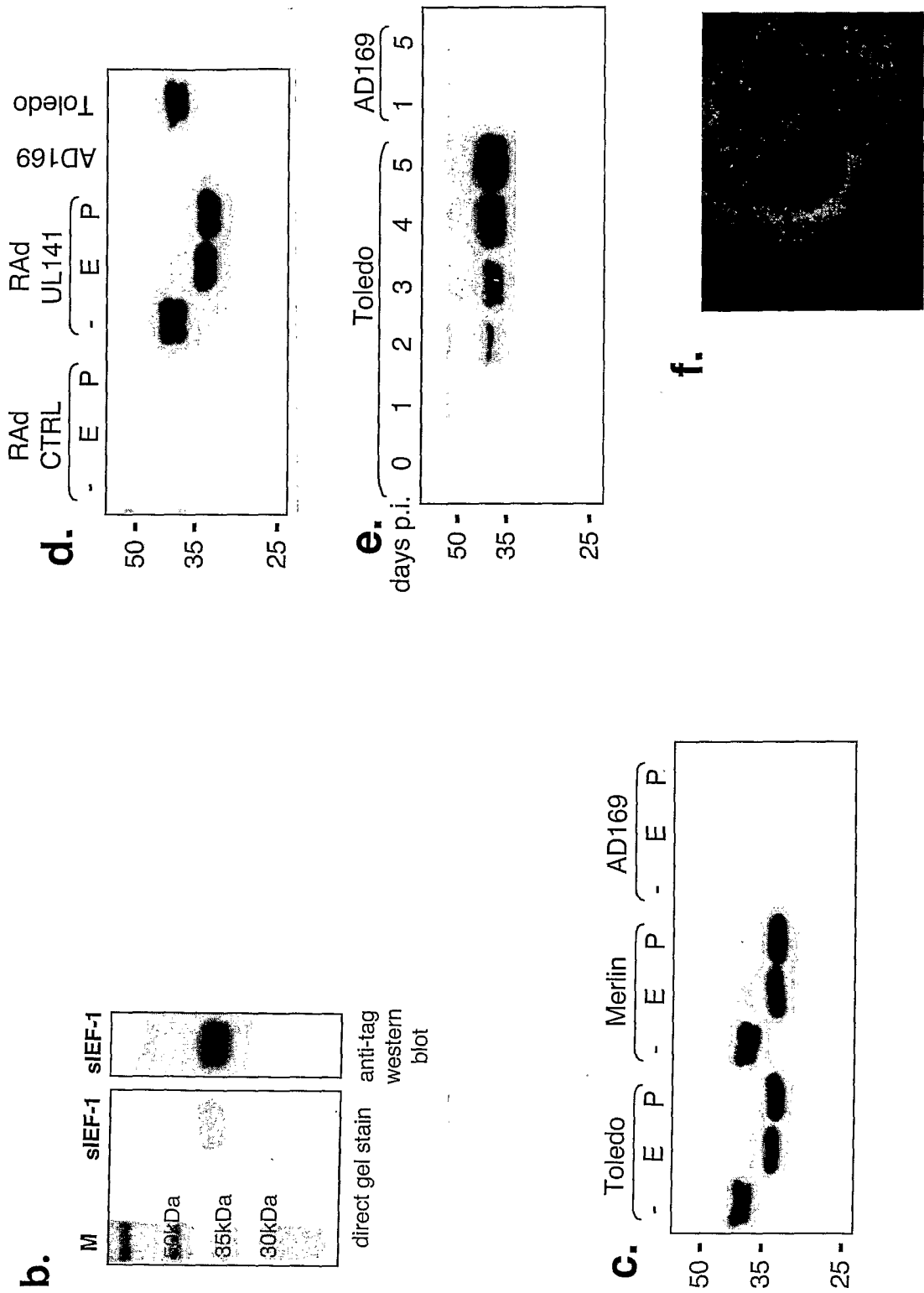


FIGURE 4a

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Fig 5

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CCMV UL14  MAG..AIRLWLPAAVAAPTGRCCDGCWCPPILRSPCLQMLSDR NRERRLSS
HCMV UL14  MGGGRLLPPLWLPL...LIAWSEWGNCC..LDAPPVVRSPCLQPVDR NRERNPGS
CCMV UL141  MARROVGKVRRLPLLTUVGMILLEPEKSSRAGALLSDAAAPTGLPKETRNVEIVSPA
HCMV UL141  MCRRESRLTPWLFVLLSCPRLLSEYSSSPFATAIAEKMW.....AE NYETTSPA

CCMV UL14  PKMLPYGDRLDVSCIFPS HAWPEVSI HVRLCYW..PEIVRS LVVDAHGQQLLHNDVTCQ
HCMV UL14  PQLLPYGDRLEVACIFPA HDWPEVSI RVHLCYW..PEIVRS LVVDARQGQVLHNDASCY
CCMV UL141  PMLVAIGERVTVPCVTMIHQWRLAVI RATFCLSPPTERSVE LVFNLSRGGEVVVPLEEKL
HCMV UL141  PVLVAEGEQVTIPCTVMTFHSWPMV9 RARFCRS..HDGSDE LILD AVKGHRLMNGLQYR

CCMV UL14  SSGR.....WS FEDGGAQAQTLLSFRLITQT AGTYHCVLGNETHGVTESTALVADVHI
HCMV UL14  AGGR.....WR FEDGGAQAQRLEBSFRLITET AGTYTCVLGNETHSLATETTALVADVHI
CCMV UL141  TESRLRGGSYNFTDLHVAHLF1TFERATASSAGVECLLRNDSHALVLQRFVVLTHLET
HCMV UL141  PYAT.....WN FSQHLGQIFSLTFNVSMDAQMECEVLRNYSHGLIMQRFVILTQLET

CCMV UL14  LQKTDR.S CDLAFGSRQTQKYIWPDP5KLSRDCQWEGER..HRLVHYI PGSSGE....
HCMV UL14  LRHSDR.S CDLAFGSRSQTRYLWTPDP5RLRSIN CQWEGER..HRVVHYI PGTSGL....
CCMV UL141  LSWADEPCCTPPLGRHSQDRQWSPTPWRLKHVDEGWSSQMGRNRFYI EPASIGDGTGTE
HCMV UL141  LSRPDEPCCTPALGRYSLGDQW5PTPWRLRNHDEG.TYRGFQ RNYFYI .....

CCMV UL14  .....E PSCEENEH.QLCVPFIWKSADNNCSRODREDEEEDDEDPSSTA/
HCMV UL14  .....L PSCEEDER.EL CVPFISQSIADNNCSRRHRVDGARRR.....
CCMV UL141  INIDAGEGEDEECWPLCQPEEQPDQWPVVRQYRLPGYRSGDKQAKFVPALPTEPP
HCMV UL141  .....GRADAEDCWK PAC.PD EEPDRCWTVIQRYRLPGDEYRSQPHPPKFLPVT.....

CCMV UL14  KQPSSQLSRDWPSPDRI QVLAACS VFTTTVHLLCYMCS KSYRRLTDEGEELYRRC
HCMV UL14  .....YHLRRDY WTDPKLGLAAGSVALTS LCHLLCYMCS ESYRRLNTEEESEAAEET
CCMV UL141  LATPPEIPTGLSP WATRGVASF LGFWGFSVGVFCYLI YLHCCRR.C....LGRR.GDGY
HCMV UL141  .APPADIDTGMSPWATRG1 AAF LGFW51 FTVCFLCYLCYLOCCGWCPTGRGRGGEG

CCMV UL14  VAPETEESDRPGRDKGDEKSDPSSHLKDE
HCMV UL14  AAGEASAVAAAAVSEEEQRRR
CCMV UL141  RRLPQYDEYPGHRMKT
HCMV UL141  RRLPTYDSYPCVVRKMKR

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FIGURE 5

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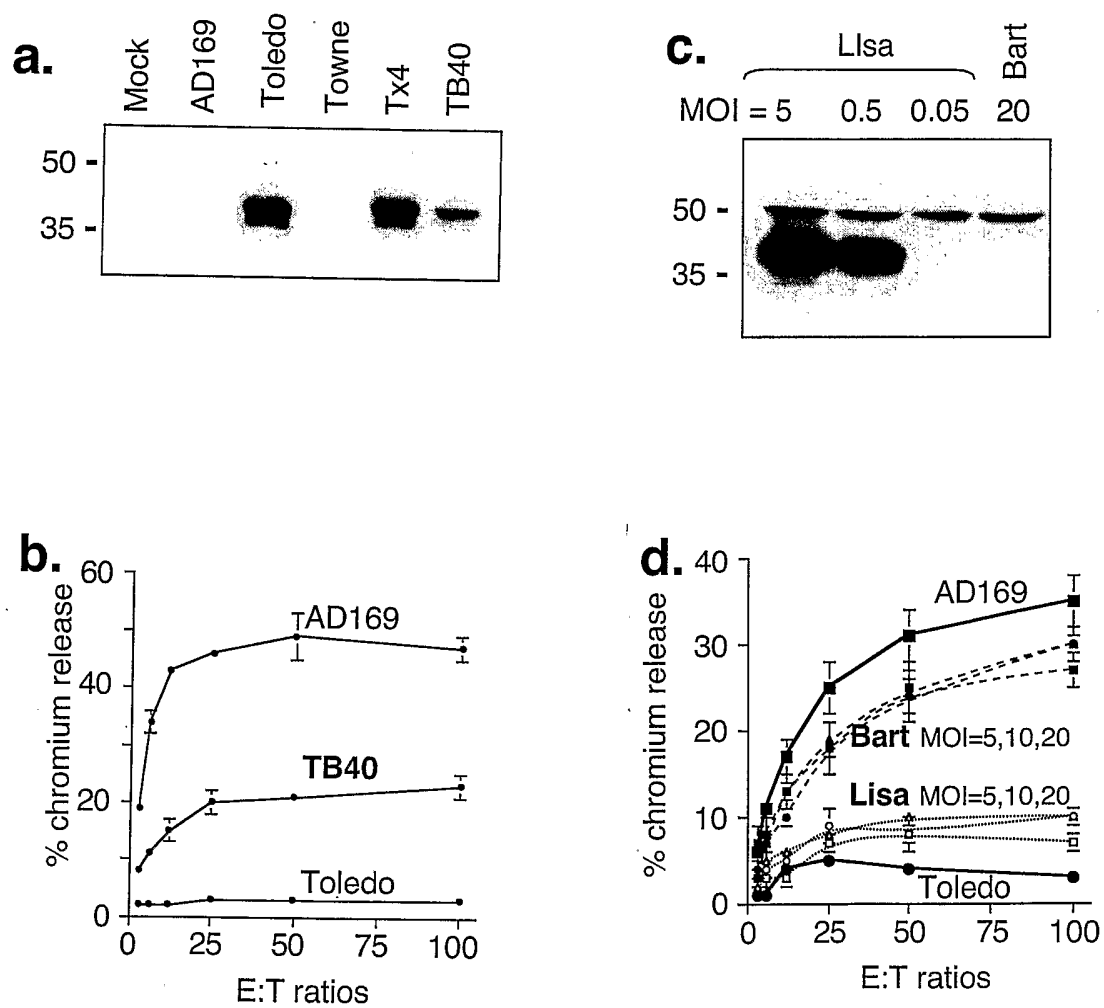


FIGURE 6

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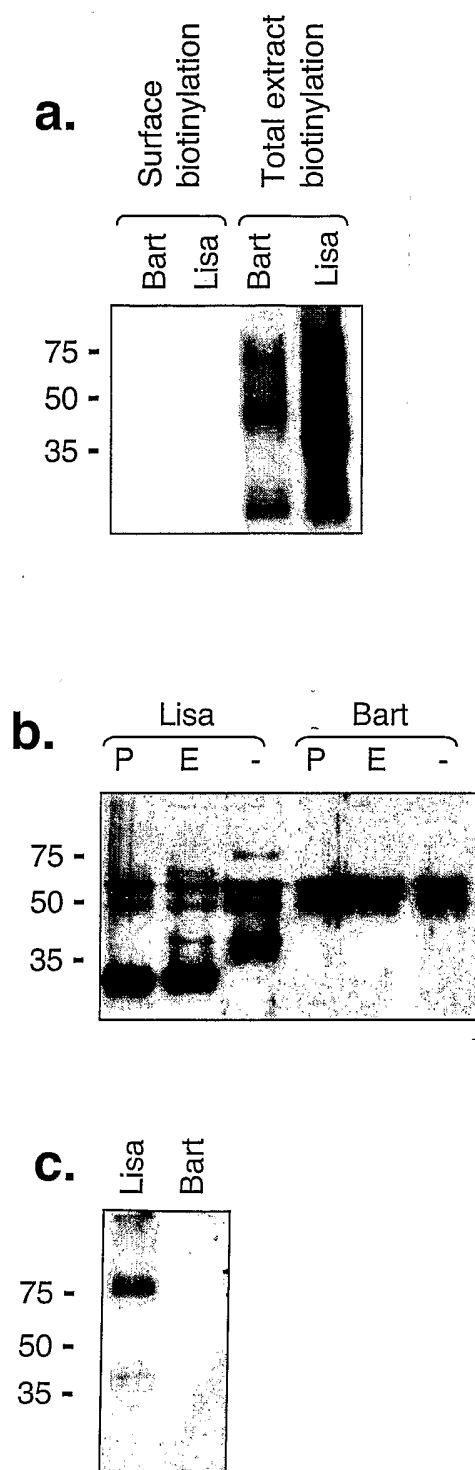


FIGURE 7