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(54) Title: CHIMERIC DR5 POLYPEPTIDES AND USES THEREOF

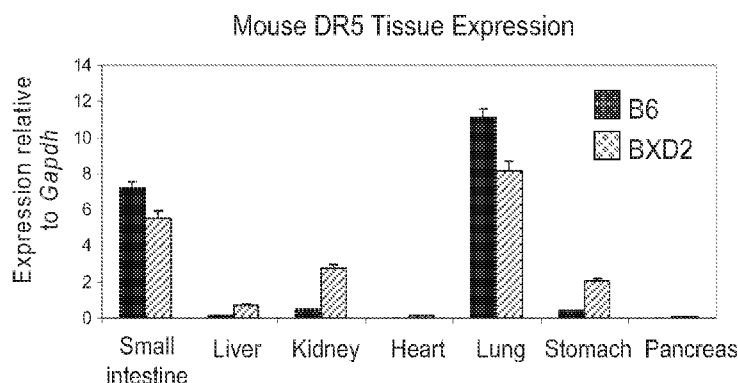


FIG. 1

(57) Abstract: Provided herein are chimeric DR5 polypeptides and nucleic acids encoding the chimeric DR5 polypeptides. Also provided are expression vectors and cells comprising the chimeric DR5 nucleic acids or polypeptides. Transgenic animals comprising one or more cells expressing the chimeric DR5 polypeptides are provided. Also provided are methods of screening for an agent or a combination of agents with DR5-mediated apoptosis activity.



CHIMERIC DR5 POLYPEPTIDES AND USES THEREOF

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 61/240,838, filed September 9, 2009, which is incorporated by reference herein in its entirety.

BACKGROUND

In a variety of physiological and pathological processes, abnormal cells are deleted by programmed cell death referred to as apoptosis. Insufficient apoptosis leads to the development of many diseases including cancer and autoimmune diseases. Enhanced apoptosis by apoptosis-inducing ligands such as agonistic antibodies are considered potential therapeutic strategies for eliminating abnormal cells. DR5 expression is up-regulated in tumor tissues compared to normal tissues. Cancer cells can be therapeutically targeted using, for example, DR5 antibodies that promote apoptosis. Such treatment ideally leads to apoptosis of these target cells without systemic toxicity (Yada et al., Ann. Oncol. 19:1060-7 (2008)). DR5 is also upregulated on rheumatoid arthritis synovial fibroblasts, which can also be therapeutically targeted using DR5 antibodies (Ichikawa et al., J. Immunol. 171:1061-9 (2003)).

SUMMARY

Provided herein are chimeric DR5 polypeptides and nucleic acids encoding the chimeric DR5 polypeptides. Also provided are expression vectors, transgenes, and cells comprising the chimeric DR5 nucleic acids or polypeptides. Transgenic animals with one or more cells comprising the chimeric DR5 nucleic acids are provided. Also provided are methods of screening for an agent or a combination of agents with DR5-mediated apoptosis activity using the provided polypeptides, nucleic acids, vectors, transgenes, cells, and transgenic animals.

The details of one or more aspects are set forth in the accompanying drawings and the description below. Other features, objects, and advantages will be apparent from the description and drawings, and from the claims.

DESCRIPTION OF DRAWINGS

Figure 1 is a histogram showing the expression levels of endogenous mouse DR5 mRNA in B6 and autoimmune BXD2 mouse tissues.

Figure 2 shows increased expression of DR5 on autoreactive B-cells in BXD2 mice. The top, left panel is an image of an agarose gel showing reverse transcriptase polymerase chain reaction (RT-PCR) analysis of DR5 expression in CD4⁺ T-cells and B-cells in B6 and BXD2 mice. The top, right panel is an image of an agarose gel showing RT-PCR analysis of DR5 and hTNFI (last lane), in mouse synovial fibroblasts (MSF) in B6, arthritis BXD2 and arthritis human TNFI transgenic mice. The bottom, left panel is a histogram showing quantitation of DR5 expression relative to expression of 18S RNA. The bottom, right panel is a histogram showing quantitation of DR5 and hTNFI expression relative to expression of 18S RNA.

Figure 3 is a histogram showing that the DR5 antibody, TRA-8, enhances apoptosis of human CD4 T-cells (left) and CD19 B-cells (right) from human lupus patients.

Figure 4 is an alignment of the amino acid sequences of human DR5 (SEQ ID No:5) and mouse DR5 (SEQ ID NO:4). Figure 4 also shows the transmembrane and death domains of DR5 and the location of the junctions of the chimeric DR5 constructs.

Figure 5 is a Western blot showing the expression of various chimeric DR5 polypeptides under control of the cytomegalovirus (CMV) promoter or the mouse DR5 1 kilobase (kb) promoter in transiently transfected HEK293 cells.

Figure 6 are photomicrographs showing the viability of mouse NIH-3T3 cells expressing various chimeric DR5 polypeptides.

Figures 7A and 7B are histograms showing the viability of mouse NIH-3T3 cells (Figure 7A) or mouse B16 melanoma cells (Figure 7B) transfected with a construct with a CMV promoter driven human DR5, mouse DR5 or chimeric DR5 with and without the addition of TRA-8. Data also includes results from NIH-3T3 cells transfected with promoterless mouse DR5 and green fluorescent protein under the control of the CMV promoter.

Figure 8 is a histogram showing the viability of human HEK293 cells expressing human DR5 or chimeric DR5 under control of the CMV promoter with

and without the addition of TRA-8. Data also include the results from HEK293 cells treated with lipofectamine only (“Mock”).

Figure 9 is a schematic showing various chimeric DR5 constructs with different regulatory elements.

5 Figure 10 is a histogram showing the percentage of cell surface expression of chimeric DR5 in mouse NIH-3T3 cells using various chimeric DR5 constructs.

Figure 11 is a histogram showing the effects of various chimeric DR5 constructs with different regulatory elements on the viability of mouse NIH-3T3 cells.

10 Figure 12 is a FACS analysis showing TRA-8 enhanced apoptosis of mouse NIH-3T3 cells expressing chimeric DR5.

Figure 13 is a schematic showing *Floxed*-STOP-chimeric DR5 constructs.

Figure 14 are FACS analyses showing *Floxed*-STOP prevents the expression of DR5 in transfected cells.

15 Figure 15 are photomicrographs showing the *Floxed*-STOP can be removed by expression of Cre.

Figure 16 is a histogram showing *Floxed*-STOP enhanced the viability of mouse B16 melanoma cells expressing chimeric DR5.

Figure 17 is a histogram showing the effects of *Floxed*-STOP chimeric DR5 on early embryonic development.

20 Figure 18 is a schematic showing *Floxed*-STOP chimeric DR5 constructs with anti-apoptotic elements.

Figure 19 is a graphic map of the 3kb mouse DR5 promoter-*Floxed*-c-FLIP-STOP-chimeric DR5 construct.

25 Figure 20 is a histogram showing the effects of the 3kb mouse DR5 promoter-*Floxed*-c-FLIP-STOP-chimeric DR5 on early embryonic development.

Figure 21 is an image of a gel showing integration of transgenic DNA in the pre-implantation embryos as determined by nested PCR.

30 Figure 22 shows the genotyping of five (5) 3kb/FLIP/STOP/Chimeric DR5 transgenic mice. Primers were targeted to the human portion of the chimeric DR5 nucleic acid sequence.

Figure 23 shows the genotyping of thirteen (13) 3kb/FLIP/STOP/Chimeric DR5 transgenic mice. Primers were targeted to the human portion of the chimeric DR5 nucleic acid sequence.

Figure 24 shows the comparison of human, mouse and chimeric DR5. Figure 24A shows amino acid alignment of human and mouse DR5. Figure 24B shows a schematic diagram of human, mouse and chimeric DR5. Figure 24C shows the expression of cell surface human DR5 recognized by anti-human DR5 antibody in mouse NIH-3T3 cells after transiently transfection with the indicated constructs as determined by flow cytometry analysis. Figure 24D shows the confirmation of human DR5 expression and its molecular mass as determined by Western blot analysis.

Figure 25 shows the DR5/TRA-8 mediated cell killing and approach to generate human/mouse chimeric DR5 transgenic mice. Figures 25A and 25B show DR5/TRA-8 mediated killing in HEK293 (Fig. 25A) and NIH-3T3 (Fig. 25B) cells transiently transfected with human, mouse, and chimeric DR5. Figure 25C shows a schematic representing the elements of the construct used for generating chimeric DR5 transgenic mice. The human mouse chimeric DR5 is driven by mouse 3kb death receptor putative promoter. A Floxed- FLIP_L STOP cassette is introduced between the promoter and the chimeric DR5, which can be removed by crossing the DR5 transgenic mouse strain to Cre-expressing mouse strain. Figure 25D shows real-time PCR analysis of the chimeric DR5 expression on the indicated tissues from the Ubc.CreDR5 double positive transgenic mice after induction of Cre expression by Tamoxifen. Expression is represented as the ratio of copy numbers of chimeric DR5 to that of GAPDH.

Figure 26 shows that TRA-8 treatment prevents the development and attenuates the severity of arthritis in Ubc.CreDR5 mice. Arthritis was induced on Ubc.CreDR5 mice via intradermal injection of chick type II collagen emulsified in CFA and an immunofluorescence assay (IFA) was performed on day 0 and day 32 (arrows). TRA-8 treatment was initiated on day 0 and day 30 for early and late treatment group respectively (arrow heads). Clinical score (0-3 per paw; n=6 per group) were followed daily until the mice were sacrificed (Fig. 26A). Figure 26B shows representative H&E and Mac-3 immunohistochemistry (IHC) staining of the

knee joint sections from DR5 Tg⁺ or DR5⁻ wild-type mice treated with TRA-8. The expression of human DR5 in different populations of immune cells was determined by flow cytometry analysis. Figure 26C shows representative histograms showing the percentage of human DR5. Figure 26D shows a histogram demonstrating the average of the indicated population of cells expressing human DR5. Figures 26E and 26F show the Percentage of CD11b^{high} spleen macrophages from Ubc.CreDR5 mice that were untreated, treated with TRA-8 at early time point, or treated with TRA-8 at late time point as determined by FACS analysis. Figure 26G shows a histogram demonstrating the proliferation of LPS-stimulated spleen cells from Ubc.CreDR5 and Ubc.Cre mice as determined by a [³H]-thymidine incorporation assay.

Figure 27 shows that TRA-8 treatment ameliorates the collagen II-induced arthritis (CIA) condition in Lyz.CreDR5 mice. CIA was induced on Lyz.CreDR5 mice via intradermal injection of chick type II collagen. TRA-8 treatment was initiated on day 28. Clinical score were followed daily until the mice were sacrificed (Figure 27A). Figure 27B is a representative image showing cathepsin-B activity in joints measured by *in vivo* imaging using a NIRF probe, ProSense 750. Figures 27C and 27D show FACS analysis of cell surface expression of chimeric DR5 on CD11b⁺ spleen cells (Fig. 27C) and CD11b⁺ cell (Fig. 27D) depleted by TRA-8. Figure 27E shows histological assessment of representative knee joints from either DR5 Tg⁺ or control DR5⁻ wild type mice injected with TRA-8.

Figure 28 shows that TRA-8 treatment induces apoptosis of macrophages in fresh synovial fragments isolated from human synovial fluid. Figure 28A shows two major component cells of synovial lining layer, macrophages and fibroblasts in human rheumatoid arthritis (RA) and osteoarthritis (OA), revealed by confocal microscope using anti-CD68 and anti-CD90 antibodies, respectively. Figure 28B shows cell surface expression of DR5 on macrophages in synovial lining layer from RA patients recognized by TRA-8. Figure 28C shows cell death of the synovial fragments before and after TRA-8 treatment analyzed by 7-AAD staining. Figures 28D and 28E show apoptosis induced by TRA-8 on macrophages isolated from RA synovial fragments confirmed by cell morphology (Fig. 28D) and Annexin-V FACS analysis (Fig. 28E).

DETAILED DESCRIPTION

Provided herein are chimeric DR5 polypeptides (e.g., human/mouse chimeric DR5 polypeptides) and fragments, as well as, nucleic acids, vectors, transgenes, and animal models expressing chimeric DR5. These polypeptides, fragments, nucleic acids, vectors, transgenes, and animal models can be used to evaluate the effect of DR5 agonists or antagonists, for example DR5 antibodies. The animal models can also be used to develop models of cancer and inflammatory arthritis and other autoimmune diseases.

As described in the Examples below, the tissue and cellular expression of DR5 in normal B6 and autoimmune BXD2 mice has been determined. Various human/mouse (hu/mo) chimeric DR5 molecules have been produced that express the human DR5 extracellular domain with a mouse transmembrane and mouse intracellular domain of DR5. Transient expression of hu/mo DR5 in both human and mouse cells has been verified and enables TRA-8 mediated apoptosis in transfected cells. The effect of various lengths of mouse DR5 promoter, first intron and 3' UTR on the expression of chimeric DR5 has been determined. *Floxed*-STOP-Chimeric DR5 constructs have been developed to enable spatial and temporal regulation of DR5 expression and to test the efficacy of the temporal expression of DR5 and the TRA-8 mediated apoptosis in transfected cells. Two new *Floxed*-STOP-Chimeric DR5 constructs, in which the anti-apoptotic gene, cFLIP or cIAP2 was embedded upstream of the STOP element and between two LoxP sites have been generated. This approach confers the anti-apoptotic protective effect until the *Floxed*-(cFLIP/cIAP2) STOP is excised and the chimeric DR5 is turned on in the Tg mice. Additionally, transgenic mice containing the *Floxed*-cFLIP-STOP-Chimeric DR5 transgene have been made.

Further, transgenic mice containing the *Floxed*-STOP hu/mo chimeric DR5 molecule have been made and crossed with tamoxifen inducible ubiquitin Cre (Ubc.Cre) transgenic mice to produce Ubc.Cre DR5 transgenic mice. These mice were induced to develop collagen II-induced arthritis (CIA), and treatment with TRA-8 inhibited the development of CIA. The *Floxed*-STOP hu/mo chimeric DR5 transgenic mice were also crossed with Lyz.Cre transgenic mice to produce DR5 Lyz.Cre transgenic mice to achieve restricted expression of the chimeric DR5

molecule in myeloid lineage cells. Treatment with TRA-8 reduced the number of macrophages in the transgenic mice and inhibited the severity of arthritis in mice induced to develop arthritis.

Provided herein are chimeric DR5 polypeptide sequences. The chimeric DR5 polypeptide sequence contains (i) the extracellular domain of human DR5 and (ii) the intracellular domain of a non-human DR5. Optionally, the chimeric DR5 polypeptide sequence further contains the transmembrane domain of a non-human DR5.

Optionally, the non-human DR5 is mouse DR5. The transmembrane domain and the intracellular domain are from the same or different species. Optionally, the

transmembrane domain and intracellular domain are from a mouse DR5. Optionally, the extracellular domain of human DR5 comprises SEQ ID NO:15, the transmembrane domain of mouse DR5 comprises SEQ ID NO:16, and/or the intracellular domain of mouse DR5 comprises SEQ ID NO:17. Optionally, the

chimeric DR5 polypeptide contains linker sequences between the domains. By way

of example, chimeric DR5 polypeptide sequences comprise SEQ ID NO:1, SEQ ID NO:2 or SEQ ID NO:3. There are a variety of sequences for DR5 from various

species that are disclosed on Genbank and these sequences and others are herein incorporated by reference in their entireties as well as for individual subsequences

contained therein. For example, the amino acid sequence for rat DR5 can be found at Genbank Accession No. ACL51000, and the nucleic acid sequence for rat DR5 can be found at Genbank Accession No. NM_001108873. Further, the amino acid sequences for mouse DR5 and human DR5 are provided in SEQ ID NOs:4 and 5, respectively.

Further contemplated herein are modified versions of the sequences or subsequences, including, for example, sequences with one or more insertions,

deletions or substitutions including conservative and/or non-conservative substitutions. Also contemplated herein are modified versions of the sequences with 85, 90, 95, or 99% identity to the disclosed sequences and subsequences.

Furthermore, the chimeric polypeptides can include sequences between the various domains (including, e.g., linker sequences). By way of example, linker polypeptides include alternating Serine and Glycine stretches exemplified by (GGGGS)₃ (SEQ ID NO:19) (Freund et al., FEBS Lett. 320:97-100 (1993)), (GSGSGS)_n (SEQ ID NO:20), and G(SGGG)₂SGGT (SEQ ID NO:21); the flexible linker peptide of *Trichoderma*

reesei cellobiohydrolase I (CBHI) (Takkinen et al., Prot. Eng. 4:837-41 (1991)); and elbow-like peptides such as SAKTTP (SEQ ID NO:22), RADAAP (SEQ ID NO:23), and derivatives thereof (see Le Gall et al., Prot. Eng. Des. Sel. 17:357-366 (2004), which is incorporated herein by reference in its entirety for all derivatives and methods of making same).

The chimeric DR5 polypeptide optionally contains sequences encoding a purification tag such as a GST tag or HIS tag.

As used herein, the term peptide, polypeptide, protein or peptide portion is used broadly herein to mean two or more amino acids linked by a peptide bond. Protein, peptide and polypeptide are also used herein interchangeably to refer to amino acid sequences. It should be recognized that the term polypeptide is not used herein to suggest a particular size or number of amino acids comprising the molecule and that a peptide described herein can contain up to several amino acid residues or more.

As used herein, the term chimeric peptide, polypeptide, protein or peptide portion refers to a peptide, polypeptide, protein or peptide portion containing two amino acid sequences or subsequences from two different species. For example, provided are chimeric polypeptides containing a first amino acid sequence from human DR5 and a second amino acid sequence from non-human DR5.

As with all peptides, polypeptides, and proteins, it is understood that conservative modifications in amino acid sequences can occur that do not alter the nature or function of the peptides, polypeptides, or proteins. For example, amino acid sequence modifications typically fall into one or more of three classes: substitutional, insertional or deletional variants. Insertions include amino and/or carboxyl terminal fusions as well as intrasequence insertions of single or multiple amino acid residues. Insertions ordinarily will be smaller insertions than those of amino or carboxyl terminal fusions, for example, on the order of one to four residues. Deletions are characterized by the removal of one or more amino acid residues from the protein sequence. Typically, no more than about from 2 to 6 residues are deleted at any one site within the protein molecule. These variants ordinarily are prepared by site specific mutagenesis of nucleotides in the DNA encoding the protein, thereby producing DNA encoding the variant, and thereafter expressing the DNA in

recombinant cell culture. Techniques for making substitution mutations at predetermined sites in DNA having a known sequence are well known, for example M13 primer mutagenesis and PCR mutagenesis. Amino acid substitutions are typically of single residues, but can occur at a number of different locations at once; 5 insertions usually will be on the order of about from 1 to 10 amino acid residues; and deletions will range about from 1 to 30 residues. Deletions or insertions optionally are made in adjacent pairs, i.e., a deletion of 2 residues or insertion of 2 residues. Substitutions, deletions, insertions or any combination thereof may be combined to arrive at a final construct. The mutations must not place the sequence out of reading 10 frame and preferably will not create complementary regions that could produce secondary mRNA structure. Substitutional variants are those in which at least one residue has been removed and a different residue inserted in its place. Such substitutions generally are made in accordance with the following Table 1 and are referred to as conservative substitutions.

TABLE 1:Amino Acid Substitutions

Amino Acid	Substitutions (others are known in the art)
Ala	Ser, Gly, Cys
Arg	Lys, Gln, Met, Ile
Asn	Gln, His, Glu, Asp
Asp	Glu, Asn, Gln
Cys	Ser, Met, Thr
Gln	Asn, Lys, Glu, Asp
Glu	Asp, Asn, Gln
Gly	Pro, Ala
His	Asn, Gln
Ile	Leu, Val, Met
Leu	Ile, Val, Met
Lys	Arg, Gln, Met, Ile
Met	Leu, Ile, Val
Phe	Met, Leu, Tyr, Trp, His
Ser	Thr, Met, Cys
Thr	Ser, Met, Val
Trp	Tyr, Phe
Tyr	Trp, Phe, His
Val	Ile, Leu, Met

As used herein, modification with reference to a polynucleotide or polypeptide, refers to a naturally-occurring, synthetic, recombinant, or chemical change or difference to the primary, secondary, or tertiary structure of a polynucleotide or polypeptide, as compared to a reference polynucleotide or

polypeptide, respectively (e.g., as compared to a wild-type polynucleotide or polypeptide). Modifications include such changes as, for example, deletions, insertions, or substitutions. Polynucleotides and polypeptides having such modifications can be isolated or generated using methods well known in the art.

5 Nucleic acids that encode the aforementioned amino acid sequences are also disclosed. These sequences include all degenerate sequences related to a specific protein sequence, i.e., all nucleic acids having a sequence that encodes one particular protein sequence as well as all nucleic acids, including degenerate nucleic acids, encoding the disclosed variants and derivatives of the protein sequences. Thus, while
10 each particular nucleic acid sequence may not be written out herein, it is understood that each and every sequence is in fact disclosed and described herein through the disclosed protein sequence. Thus, provided are nucleic acid sequences encoding a chimeric DR5 polypeptide. The nucleic acid sequence may comprise untranslated regions at either end or internally. The chimeric DR5 polypeptide contains (i) the
15 extracellular domain of human DR5 and (ii) the intracellular domain of non-human DR5. Optionally, the chimeric DR5 polypeptide further contains the transmembrane domain of a non-human DR5. Optionally, the non-human DR5 is mouse DR5. Optionally, the extracellular domain of human DR5 comprises SEQ ID NO:15, the transmembrane domain of mouse DR5 comprises SEQ ID NO:16, and/or the
20 intracellular domain of mouse DR5 comprises SEQ ID NO:17. Optionally, the chimeric DR5 polypeptide includes linker sequences between the domains. Optionally, the chimeric DR5 polypeptide comprises SEQ ID NO:1, SEQ ID NO:2 or SEQ ID NO:3. Optionally, the nucleic acid sequence is SEQ ID NO:9. The nucleic acid sequences provided herein are examples of the genus of nucleic acids and are not
25 intended to be limiting.

Further contemplated herein are modified versions of the nucleic acid sequences or subsequences, including, for example, sequences with one or more insertions, deletions or substitutions. Also contemplated herein are modified versions of the nucleic acid sequences with 85, 90, 95, or 99% identity to the disclosed
30 sequences and subsequences. Furthermore, the chimeric polypeptides can include intervening sequences between the various domains, the intervening sequences

encoding, for example, sequences encoding linker sequences or untranslated sequences.

Those of skill in the art readily understand how to determine the relative identity of two proteins or nucleic acids. For example, the identity can be calculated after aligning the two sequences so that the identity is at its highest level. Another way of calculating identity can be performed by published algorithms. Optimal alignment of sequences for comparison may be conducted by the local identity algorithm of Smith and Waterman, *Adv. Appl. Math.* 2:482 (1981); by the identity alignment algorithm of Needleman and Wunsch, *J. Mol Biol.* 48:443 (1970); by the search for similarity method of Pearson and Lipman, *Proc. Natl. Acad. Sci. USA* 85:2444 (1988); by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI); or by inspection.

The same types of identity can be obtained for nucleic acids by for example the algorithms disclosed in Zuker, *Science* 244:48-52 (1989); Jaeger et al., *Proc. Natl. Acad. Sci. USA* 86:7706-10 (1989); Jaeger et al., *Methods Enzymol.* 183:281-306 (1989), which are herein incorporated by reference for at least material related to nucleic acid alignment. It is understood that any of the methods typically can be used and that in certain instances the results of these various methods may differ, but the skilled artisan understands if identity is found with at least one of these methods, the sequences would be said to have the stated identity, and be disclosed herein.

Also provided are expression vectors, such as transgenes, comprising these nucleic acids, wherein the nucleic acids are operably linked to an expression control sequence. Thus, provided is an expression vector containing a first nucleic acid sequence encoding a chimeric DR5 polypeptide. Optionally, the first nucleic acid sequence is SEQ ID NO:9. Optionally, the expression vector further includes a promoter sequence operably linked to the first nucleic acid sequence. Optionally, the promoter sequence comprises SEQ ID NO:6, SEQ ID NO:7, or SEQ ID NO:8. Optionally, the expression vector contains a second nucleic acid sequence encoding the first intron of a mouse DR5 polypeptide. Optionally, the second nucleic acid sequence is located 3' to the promoter sequence and 5' to the first nucleic acid sequence. Optionally, the expression vector contains regulatory elements, for

example, a *Floxed*-STOP element. Optionally, the expression vector comprises SEQ ID NO:14. Optionally, the vector further contains sequences that encode anti-apoptotic elements, for example, FLICE inhibitory protein (FLIP) or cellular inhibitor of apoptosis (cIAP2).

5 Optionally, the nucleic acids, constructs, expression vectors and transgenes provided herein further comprise a sequence encoding a reporter molecule.

Optionally, the reporter molecule is green fluorescent protein (GFP), yellow fluorescent protein (YFP), cyan fluorescent protein (CFP), red fluorescent protein (RFP), chloramphenicol acetyl transferase (CAT), or luciferase.

10 Further provided are cells comprising the provided expression vectors or nucleic acids. Optionally, the provided nucleic acid sequences are located in the genome of the cell. Such expression vectors and cultured cells can be used to make the disclosed polypeptides. Optionally, the cells are embryonic stem cells, gametes (e.g., an egg), cancer cells, T-cells, B-cells, macrophages or fibroblasts.

15 Also provided are methods of making a cell expressing a chimeric DR5 polypeptide comprising transforming the cell with a vector or nucleic acid encoding chimeric DR5. Optionally, the cell is transformed with a vector or nucleic acid sequence comprising SEQ ID NO:9 or SEQ ID NO:14. Optionally, the vector is an expression vector or an integration vector. There are a number of compositions and
20 methods that can be used to deliver nucleic acids to cells, either *in vitro* or *in vivo*, for example, by expression vectors. These methods and compositions can largely be broken down into two classes: viral based delivery systems and non-viral based delivery systems. For example, the nucleic acids can be delivered through a number of direct delivery systems such as, electroporation, lipofection, calcium phosphate
25 precipitation, plasmids, viral vectors, viral nucleic acids, phage nucleic acids, phages, cosmids, or by transfer of genetic material in cells or carriers such as cationic liposomes. Such methods are well known in the art and readily adaptable for use with the compositions and methods described herein. Further, these methods can be used to target certain diseases and cell populations by using the targeting characteristics of
30 the carrier.

 Also provided are transgenic animals comprising one or more cells expressing a chimeric DR5 polypeptide. As used herein, the term animal refers to non-human

animals, including, mammals, amphibians and birds. Specifically, examples include sheep, feline, bovines, ovines, pigs, horses, rabbits, guinea pigs, mice, hamsters, rats, non-human primates, and the like. The chimeric DR5 polypeptide comprises (i) the extracellular domain of human DR5 and (ii) the intracellular domain of a non-human DR5. Optionally, the chimeric DR5 polypeptide further comprises a transmembrane domain of a non-human DR5. Optionally, the extracellular domain comprises SEQ ID NO:15, the intracellular domain comprises SEQ ID NO:17, and/or the transmembrane domain comprises SEQ ID NO:16. Optionally, the chimeric DR5 polypeptide comprises SEQ ID NO:1, SEQ ID NO:2 or SEQ ID NO:3. Optionally, the genome of the cells of the transgenic animal comprises SEQ ID NO:14. Optionally, the cells of the transgenic animal expressing the chimeric DR5 polypeptide are cancer cells, T-cells, B cells, macrophages or fibroblasts. Such cells are optionally isolated from the transgenic animal. Optionally, the animal is a model for cancer, arthritis or autoimmune disease. Optionally, the arthritis is rheumatoid arthritis.

The intracellular domain and the transmembrane domain of the chimeric DR5 polypeptide are optionally from the same species as the transgenic animal. For example, if the transgenic animal is a mouse, the intracellular and transmembrane domains are from mouse DR5. If the transgenic animal is a rat, the intracellular and transmembrane domains are from rat DR5.

Animal models for cancer, arthritis, or autoimmune disease can, for example, include genetic knockouts, wherein the function of a gene or multiple genes is lost due to targeting the gene or multiple genes, and genetic knock-ins, wherein a gene or multiple genes of interest are targeted specifically or non-specifically into the genome of the animal to express the gene or genes of interest. Examples of animal models of cancer that contain a genetic knockout or knock-in include, for example, knockouts or knock-ins of cell cycle regulators (e.g., cyclin dependent kinases, cyclins, and mitogen activated protein kinases); components of signaling pathways (e.g., growth factors, growth factor receptors, cytokines, chemokines, and adapter proteins); and regulators of gene expression (e.g., transcription factors, splicing factors, translation factors, post-transcriptional regulators (e.g., miRNAs), and post-translational regulators (e.g., kinases, phosphatases, and acetyl-transferases)). Animal models of

arthritis can, for example include, knockouts or knock-ins of genes involved in regulating inflammation (e.g., cytokines, chemokines, tumor necrosis factor alpha (TNF α), transforming growth factor beta (TGF β), and interferon gamma (IFN γ)).

Animal models of autoimmune diseases can, for example, include knockouts or
5 knock-ins of genes involved in immunity (e.g., immunoglobulins, chemokine and chemokine receptors, tumor necrosis factor receptors, co-stimulatory molecules, and cytokines and cytokine receptors). Optionally, the transgenic animal comprising one or more cells expressing a chimeric DR5 polypeptide can be crossed with an animal model of cancer, arthritis, or an autoimmune disease. Also provided herein is a
10 progeny animal resulting from a cross between a transgenic animal comprising one or more cells expressing a chimeric DR5 polypeptide and a transgenic animal model of cancer, arthritis, or an autoimmune disease. Optionally, the transgenic animal is the DBA/1-hDR5 human/mouse chimeric Tg mouse or the B6-hu/mo chimeric DR5 Tg mouse, which are described in Example 10 below. Optionally, the transgenic animal
15 is FVB.Cg-Tg(MMTV-vHaras)SH1Lcd/J-hDR5 human/mouse chimeric Tg mouse, which is described in Example 11 below. Further provided is a cell isolated from a progeny animal resulting from a cross between a transgenic animal comprising one or more cells expressing a chimeric DR5 polypeptide and a transgenic animal model of cancer, arthritis, or an autoimmune disease.

20 Animal models of cancer can, for example, be created by directly injecting tumor cells into the animal. The tumor cells can be injected subcutaneously, intraperitoneally, or intravenously. Tumor cells can be injected in a targeted manner to a specific organ or location within the animal, or, alternatively, tumor cells can be injected at random, in a non-targeted manner. Optionally, the transgenic animal
25 comprising one or more cells expressing a chimeric DR5 polypeptide is injected directly with tumor cells to create an animal model of cancer.

Animal models of cancer, arthritis, and autoimmune disease can, for example, be created by exogenously administering an agent or agents that cause cancer, arthritis, or an autoimmune disease. Examples of an agent or agents that causes
30 cancer, arthritis, or an autoimmune disease can include chemical compounds, radiation, viral infection, or bacterial infection. See Calbrese and Blain, *Toxicological Sciences* 50:169-85 (1999); Van Der Berg, *Kelley's Textbook of Rheumatology*, 8th

ed., W.B. Saunders Company, Chapter 25 (2008); and Lusdewig et al., *Experimental Physiology* 85(6):653-9 (2000). Optionally, the transgenic animal comprising one or more cells expressing a chimeric DR5 polypeptide is administered an agent or agents that cause cancer, arthritis, or an autoimmune disease. Optionally, the animal model is a DBA/1, B6 or FVB.Cg-Tg(MMTV-vHaras)SH1Lcd/J mouse. These animals are described in Examples 10 and 11 below.

As used herein, transgenic animal refers to any animal, e.g., a mammal, bird, or an amphibian, in which one or more of the cells of the animal contain a heterologous nucleic acid. The heterologous nucleic acid can be introduced using known transgenic techniques. The nucleic acid is introduced into the cell, directly or indirectly. For example, the nucleic acid can be introduced into a precursor of the cell or by way of deliberate genetic manipulation, such as by microinjection or by infection with a recombinant virus. The nucleic acid may be integrated within a chromosome, or it may be an extrachromosomally replicating DNA.

Methods for making transgenic animals using a variety of transgenes have been described in Wagner et al., *Proc. Nat. Acad. Sci. USA* 78:5016-20 (1981); Stewart et al., *Science* 217:1046-1048 (1982); Constantini et al., *Nature* 294:92-4 (1981); Lacy et al., *Cell* 34:343-58 (1983); McKnight et al., *Cell* 34:335-41 (1983); Brinster et al., *Nature* 306:332-6 (1983); Palmiter et al., *Nature* 300:611-5 (1982); Palmiter et al., *Cell* 29:701-10 (1982); and Palmiter et al., *Science* 222:809-14 (1983). Such methods are also described in United States Patent Nos. 6,175,057; 6,180,849; and 6,133,502.

The term transgene is used herein to describe genetic material that has been or is about to be artificially inserted into the genome of a mammalian cell, particularly a mammalian cell of a living animal. The transgene is used to transform a cell. Vectors for stable integration include, but are not limited to, plasmids, retroviruses and other animal viruses and YACS.

By way of example, the transgenic animal can be created by introducing a transgene construct into, for example, an embryonic stem cell, an unfertilized egg, a fertilized egg, a spermatozoon or a germinal cell containing a primordial germinal cell thereof, preferably in the embryogenic stage in the development of a non-human mammal (more preferably in the single-cell or fertilized cell stage and generally

before the 8-cell phase). The transgene can be introduced by known means, including, for example, the calcium phosphate method, the electric pulse method, the lipofection method, the agglutination method, the microinjection method, the particle gun method, the DEAE-dextran method and other such method. Optionally, the transgene is introduced into a somatic cell, a living organ, a tissue cell or other cell by gene transformation methods. Cells including the transgene may be fused with the above-described germinal cell by a commonly known cell fusion method to create a transgenic animal.

For embryonic stem (ES) cells, an ES cell line may be employed, or embryonic cells may be obtained freshly from a host, e.g., mouse, rat, guinea pig, and the like. Such cells are grown on an appropriate fibroblast-feeder layer or grown in the presence of appropriate growth factors, such as leukemia inhibiting factor (LIF). When ES cells have been transformed, they may be used to produce transgenic animals. After transformation, the cells are plated onto a feeder layer in an appropriate medium. Cells containing the construct may be detected by employing a selective medium. After sufficient time for colonies to grow, they are picked and analyzed for the occurrence of homologous recombination or integration of the construct. Those colonies that are positive may then be used for embryo manipulation and blastocyst injection. Blastocysts are obtained from 4 to 6 week old superovulated females. The ES cells are trypsinized, and the modified cells are injected into the blastocoel of the blastocyst. After injection, the blastocysts are returned to each uterine horn of pseudopregnant females. Females are then allowed to go to term and the resulting litters screened for mutant cells having the construct. By providing for a different phenotype of the blastocyst and the ES cells, chimeric progeny can be readily detected. The chimeric animals are screened for the presence of the modified gene, and males and females having the modification are mated to produce homozygous progeny transgenic animals.

Methods of screening for an agent or combination of agents for DR5-mediated apoptosis activity are provided. Optionally, the method comprises the steps of providing a transgenic animal containing one or more cells expressing a chimeric DR5 polypeptide; administering to the animal the agent or agents to be tested; and determining whether the agent or agents cause apoptosis of the cells expressing the

chimeric DR5 polypeptide. Apoptosis indicates the agent or agents have DR5-mediated apoptosis activity. Methods of determining apoptosis are known in the art and are also described in the Examples below. For example, apoptosis can be determined using the ATPLiteTM assay or annexin-V staining. Optionally, the method comprises the steps of providing cells expressing a chimeric DR5 polypeptide; contacting the cells with the agent or agents to be tested; and determining whether the agent or agents cause apoptosis of the cells, wherein apoptosis indicates the agent or agents have DR5-mediated apoptosis activity. Optionally, the agent is an antibody or small molecule.

Disclosed are materials, compositions, and components that can be used for, can be used in conjunction with, can be used in preparation for, or are products of the disclosed methods and compositions. These and other materials are disclosed herein, and it is understood that when combinations, subsets, interactions, groups, etc. of these materials are disclosed that while specific reference of each various individual and collective combinations and permutation may not be explicitly disclosed, each is specifically contemplated and described herein. For example, if a chimeric polypeptide is disclosed and discussed and a number of modifications that can be made to the chimeric polypeptide are discussed, each and every combination and permutation of the chimeric polypeptide, and the modifications that are possible, are specifically contemplated unless specifically indicated to the contrary. Likewise, any subset or combination of these is also specifically contemplated and disclosed. This concept applies to all aspects of this disclosure including, but not limited to, steps in methods of using the disclosed compositions or animals. Thus, if there are a variety of additional steps that can be performed it is understood that each of these additional steps can be performed with any specific method steps or combination of method steps of the disclosed methods, and that each such combination or subset of combinations is specifically contemplated and should be considered disclosed.

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application.

A number of aspects have been described. Nevertheless, it will be understood that various modifications may be made. Furthermore, when one characteristic or step

is described it can be combined with any other characteristic or step herein even if the combination is not explicitly stated. Accordingly, other aspects are within the scope of the claims.

Examples

5 Example 1: Expression of Human and Mouse DR5 in Different Tissue

Analysis of the hDR5 mRNA expression in the previously generated hDR5 transgenic (Tg) mice indicated that the highest levels of Tg could be detected in liver, lung, spleen, heart, kidney, and peripheral blood mononuclear cells. The distribution pattern mirrors that of the endogenous human DR5. However, the expression of the Tg at the protein levels on the cell surface was low unless the Tg mice were
10 immunized with sheep red blood cells to stimulate an immune response *in vivo*. Live imaging biodistribution analysis of the whole mouse body further showed that there were modestly increased levels of hDR5 detected in the liver and kidney of hDR5 Tg vs control wild-type mice. In mouse, the DR5 protein is also widely expressed and its transcript was previously reported to be expressed in the heart, lung, and kidney.
15

qRT-PCR analysis was carried out to determine the expression of mouse DR5 (mDR5) at the mRNA levels from different tissues in the B6 and autoimmune BXD2 mice (Fig. 1). As in human, DR5 mRNA is present in the lung and intestines of normal and autoimmune BXD2 mice.

To determine if DR5 is unregulated in T and B cells from autoimmune BXD2 mice relative to B6 mice, transcriptional expression of mouse DR5 was compared in CD4 T cells from B6 and BXD2 mice, and purified B cells from B6 and BXD2 mice. There is increased expression of DR5 on autoreactive B cells compared to BXD2 mice (Fig 2). As described herein, BXD2 mice exhibit increased generation of
20 autoreactive B cells with increased autoantibody production. These B cells form large germinal centers (GCs) in the spleen with high activation-induced cytidine deaminase (AID) expression (Hsu et al., J. Immunol. 178:5357-5365 (2007)). Therefore, increased expression of DR5 on B cells in BXD2 mice supports the concept that anti-DR5 therapy is beneficial as an anti-B cell therapy.
25

30 Mouse synovial fibroblasts exhibit abnormal features including increased proliferation, production of stromelysin, collagenase, IL-6 and RANKL that lead to erosion and inflammation. Two models of arthritis have been analyzed, the BXD2,

which develops a spontaneous erosive arthritis with synovial hyperplasia and erosion of cartilage and bone (Wu et al., *Arthritis Rheum.* 52:3257-68 (2005)). Also, the human TNF- α Tg mouse develops synovial hyperplasia and bone and cartilage erosion (Keffer et al., *EMBO J.* 10:4025-31 (1991)). Primary cell lines were
5 generated from mouse synovial fibroblasts from B6, BXD2, or human TNF- α transgenic mouse. There was very low DR5 expression in mouse synovial fibroblasts (MSF) from B6 mice (Fig. 2). However, DR5 expression was upregulated in mouse synovial fibroblasts from BXD2 mice and human TNF- α transgenic mice that both develop spontaneous arthritis (Fig 2). These results show that anti-DR5 therapy is
10 successful in targeting synovial hyperplasia in rheumatoid arthritis.

To determine if a DR5-antibody is effective in inducing apoptosis in lymphocytes from human autoimmune subjects, DR5 antibody-mediated apoptosis in both CD4 and CD19 B cells obtained from PBMCs of human lupus patients was analyzed. PBMCs were isolated and cultured either in the presence of absence of
15 TRA-8 (5 μ g/ml) at 37°C overnight. Cells were harvested and the percentage of CD4 and CD19 cells undergoing apoptosis was determined by FACS analysis. The results are shown in Figure 3.

Example 2: Generation of Human-Mouse DR5 Chimeric Constructs.

DR5 (TRAIL-R2) is one of receptors of TNF related apoptosis-inducing
20 ligand (TRAIL). The molecular structure of mouse and human DR5 consists of an extracellular domain, a transmembrane domain, and an intracellular domain which contains a death domain. The extracellular domain of human is longer than mouse (210 amino acids versus 177 amino acids), and there is 33% amino acid identity between the human and mouse forms. The intracellular domain of human is also
25 longer than mouse (202 amino acids compared to 175 amino acids), and there is 35% amino acid identity between the two (Fig. 4).

To produce a chimeric construct that exhibits the extracellular domain of human DR5 and the intracellular and transmembrane domains of mouse DR5, three chimeric proteins were produced. Chimeric protein 1 (SEQ ID NO:1) produced the
30 precise joining of the human extracellular domain with the first amino acid of the mouse transmembrane domain. See Figure 4, junction 1. Since it was not known if amino acids around the transmembrane domain were essential for proper anchoring

and orientation of the molecule, and if these are species-specific, chimeric 2 (SEQ ID NO:2) was produced such that it included 5 additional extracellular domain amino acids of the mouse, which was fused to the human extracellular domain. See Figure 4, junction 2. Because the human has 22 amino acids in the extracellular domain near the transmembrane region that are unique to human, and not found in mouse, a third construct (SEQ ID NO:3) was produced that included a membrane proximal amino acid region that was unique to mouse and is quite different in human. See Figure 4, junction 3.

Example 3: Expression of Chimeric DR5 Proteins in HEK293 Cells.

The three constructs described in Example 2 were tested for expression and function by cloning them into a pCMV/SPORT6 vector under the regulation of a strong CMV promoter. Three additional constructs were also generated by replacing the CMV promoter with the mouse DR5 1 kilobase (kb) promoter. SEQ ID NO:6 shows the sequence of the mouse DR5 1 kb promoter. The expression of these chimeric constructs at the protein levels was first tested in transiently transfected (Lipofectamine 2000) human HEK293 cells by Western blot. Chimeric construct 3 was not detected, probably due to disruption of the human extracellular domain, which is critical for the binding of anti-human DR5 antibody. Both human DR5 and chimeric construct 1 and 2 produced chimeric proteins that are of their predicted sizes (47.9, 45.5 and 45.4 kDa for human DR5, chimeric 1 and 2 respectively) (Fig 5).

The mouse DR5 1 kb promoter exhibited a low activity in human HEK293 cells (lanes 6 and 7, Fig 5). The CMV and the 1 kb mouse DR5 promoter exhibit equivalent ability to drive expression of DR5 in mouse cell lines. These results indicate that transcriptional factors or binding sites for the 1 kb promoter are different in mouse and human.

Example 4: Viability of Human and Mouse Cells After Transfection with the DR5 Constructs.

The apoptotic function of the different chimeric DR5 proteins was tested in transiently transfected (Lipofectamine 2000) NIH 3T3 cells. Mouse NIH 3T3 cells were chosen for these experiments since all chimeric proteins expressed the mouse death domain. In these experiments, signaling with anti-human DR5 was not carried out since it was known that high expression of the DR5 death domain results in

spontaneous apoptosis that does not require TRAIL or anti-DR5 signaling. The transfection efficiencies were determined by parallel transfection of a CMV-GFP vector in the NIH 3T3 cells indicating approximately 35-40% transfection efficiency. In cells transfected with chimeric DR5-1 and DR5-2, there was significant cell death by morphology (Fig. 6). In contrast, chimeric DR5-3 did not induce significant apoptosis. Therefore, chimeric 1 DR5 (SEQ ID NO:1), which consists of the full-length human extracellular domain fused precisely in frame and to the mouse transmembrane domain, was used for further experiments.

The viability of mouse NIH3T3 cells and mouse B16 melanoma cells after transfection with full-length human DR5, full-length mouse DR5, or the chimeric human/mouse DR5, was tested (Fig 7). Cell viability was determined using the ATPLite™ assay, which was carried out in a 96-well plate on a Packard TopCount (GMI, Inc., Ramsey, Minnesota). Viability was determined with and without the addition of the DR5 antibody, TRA-8. In both cell lines, transfection of the full-length human DR5 directed by CMV promoter did not result in significant spontaneous apoptosis. Further, apoptosis was not enhanced significantly by addition of TRA-8, even though this TRA-8 could bind to the extracellular domain of this full-length human DR5. Also, in both cell lines, expression of the full-length mouse DR5 induced relatively lower levels of apoptosis. Importantly, there is no additional apoptosis seen after addition of TRA-8, indicating that TRA-8 is not capable of interacting with the mouse extracellular domain and thus does not enhance the apoptosis. These experiments indicate that the anti-human DR5 does not enhance death of mouse cells transfected with full-length human or mouse DR5. Thus, the human death domain does not function properly in mouse cells.

However, cells transfected with the human-mouse chimeric DR5 exhibited a higher rate of spontaneous apoptosis, indicated by lower ATPLite™. Furthermore, addition of TRA-8 resulted in dramatic enhancement of apoptosis of chimeric DR5 in both mouse 3T3 or B6 melanoma cell lines. This result demonstrated that generation of a chimeric DR5 construct with the human extracellular domain, the mouse transmembrane and intracellular domains containing the death domain is essential for studies of TRA-8 induced apoptosis in mice.

Since the human death domain does not induce apoptosis in mouse cell lines, it was determined whether the mouse death domain could function in human cell lines. HEK293 human cells were transfected with the full-length human DR5 and the chimeric human/mouse DR5 driven by the CMV promoter. These cells enabled not only a direct comparison of the spontaneous apoptosis of the human and mouse death domains in a human cell line, but also a comparison of TRA-8 mediated apoptotic effect since both molecules include the human extracellular domain. There was equivalent viability of both the full-length human and the human mouse chimeric construct in the 293 cell lines (Fig. 8). Also, addition of TRA-8 greatly decreased the viability of both the full-length human DR5 as well as the human/mouse chimeric DR5. This result shows that the mouse death domain does have an equivalent functional effect as a human death domain in a human cell line although the human death domain does not function in mouse cell lines (Fig. 7).

Example 5: Effects of Various Lengths of the Mouse DR5 Promoter, First Intron and 3' UTR on Expression of Mouse DR5.

The regulatory regions of the human DR5 are not completely known, but both the promoter and the first intron are thought to contain critical regulatory elements (Yoshida et al., FEBS Lett. 507:381-5 (2001)). Important regulatory elements include 2 Sp1 sites in the 1 kb human promoter and several NF- κ B and p53 binding sites in the first intron of human DR5. However, there is little homology between the mouse and human promoter or the first intron. Specifically, the first intron of the human is large (25.6 kb), whereas the first intron in the mouse is much smaller (2.9 kb). The 3' UTR of human and mouse DR5 are thought to contain important regulatory elements including micro-RNA regulatory sites.

To further determine the role of the various lengths of promoter, first intron and 3' untranslated region (UTR) of mouse DR5 on DR5 expression, constructs were produced that contained either a 1 kb promoter, a 3 kb promoter, a 2.9 kb first intron, and the 3' untranslated region (Fig. 9). These constructs were tested for both expression and for the cell killing effect using FACS analysis and ATPLiteTM assay. For cell surface expression studies, the anti-human DR5 antibody (clone DJR2-4, Biolegend) was used. NIH 3T3 cells were transfected with the chimeric DR5 driven by CMV, the 1 kb promoter-DR5, the 3 kb promoter-DR5, and the 3 kb promoter plus

the first intron with and without the 3' UTR. Twenty-four to 48 hours after transfection, surface expression of the human/mouse chimeric DR5 was determined by FACS analysis.

There was high expression of the chimeric DR5 on the surface of mouse NIH 3T3 cells that were transfected either with CVM-DR5 or the 1 kb promoter-DR5. The longer 3 kb mouse DR5 promoter negatively regulated the expression, and expression was diminished further by the addition of the first intron or the first intron plus the 3' UTR (Fig. 10). These data indicated that negative regulatory elements are present within the longer promoter, the first intron and 3' UTR of mouse DR5.

To determine apoptotic function, these constructs were transfected into mouse 3T3 cell lines. TRA-8 was added 24 hours after the transfection. At 48 hours, cell viability was estimated by the ATPLite™ assay. The highest spontaneous cell death was seen with chimeric DR5 driven by the CMV promoter and it was further enhanced by the addition of TRA-8 (Fig. 11). Both the 1 kb and 3 kb DR5 promoter resulted in a lower degree of spontaneous cell death, but cell death was greatly enhanced by TRA-8.

Spontaneous cell death by the 3 kb promoter/2.9 kb first intron/DR5 or the 3 kb promoter/2.9 kb first intron/DR5/3' UTR was low, and this was not greatly enhanced by the addition of TRA-8. Apoptosis in chimeric DR5 transfected mouse 3T3 cells was also analyzed by using 7-amino-actinomycin d (7-AAD) staining and FACS analysis (Fig. 12). The results indicated that spontaneous cell death was relatively low with the 3 kb promoter transfected 3T3 cells but was significantly enhanced by addition of TRA-8. The lowest spontaneous cell death, but with the greatest enhancement by addition of TRA-8, occurred with constructs in which the human/mouse chimeric DR5 is driven by the 3 kb promoter. Therefore, the 3 kb promoter was used for future transgene construct generation.

Example 6: Tissue-Specific and Spatial/Temporal Regulation of Human/Mouse Chimeric DR5 Transgene.

It is likely that the 3 kb promoter alone does not contain sufficient regulatory elements to enable fully authentic expression of the human/mouse chimeric DR5 in all mouse tissue. Therefore, to prevent the over-expression of transgenic DR5 and to enable tissue-specific expression of DR5 (such as in T cells, B cells, or synovial

fibroblasts), a *Floxed*-STOP element was engineered between the promoter region and the chimeric DR5 (Fig 13).

To demonstrate that the *Floxed*-STOP accentuates DR5 expression in the transfected cells, the 3 kb promoter/ STOP/DR5 was compared to the 3 kb promoter/DR5 and CMV/DR5 for expression of human DR5 using FACS analysis (Fig 14). Eight percent (8%) of NIH 3T3 cells transfected with CMV/DR5 expressed hDR5, and 10% of cells transfected with the 3 kb promoter/DR5 expressed DR5. However, there was a significant decrease in the percentage of transfected cells that expressed DR5 upon transfection with the 3 kb promoter/STOP-DR5.

To determine if Cre can efficiently remove the *Floxed* STOP and enable transcription of DR5, HEK293 cells were used since they could be transfected and transfected efficiently, and, in this case, GFP was used to estimate transcription level. Approximately 20% of the cells were GFP positive (GFP+) after transfection with the CMV-IRES-GFP. In the CMV-STOP-IRES-GFP transfected cells, there were no GFP+ cells (Fig 15). However, cells transfected with the CMV-STOP-IRES-GFP and also transfected with an Ad-Cre (48-72 hours) exhibited GFP+ cells. These results showed that the *Floxed* STOP can accentuate expression of downstream genes and that the Ad-Cre can reverse it.

To further demonstrate that the *Floxed* STOP element rescues chimeric DR5 transfected cells, mouse B16 melanoma cells were transfected with either the 3 kb chimeric-DR5, the 3 kb/STOP/chimeric DR5 or control CMV/GFP. Cell viability was determined using the ATPLite™ assay. Cells transfected with a chimeric DR5 exhibited low viability (Fig 16). The viability of cells was further suppressed by addition of TRA-8 (Fig 16). However, cell viability was increased in cells transfected with the 3 kb promoter/'STOP/chimeric DR5. Importantly, addition of TRA-8 did not have an effect on viability of the 3 kb/STOP/chimeric DR5 transfected cells, suggesting that the *Floxed* STOP element prevented the expression of chimeric DR5 in the transfected B16 melanoma cells.

Example 7: Analysis of DR5 Transgenic Embryos.

DR5 is a pro-apoptotic molecule. For successful generation of DR5 Tg mice, one prerequisite is that the transgene is not embryonic lethal. Therefore, the effect of

different DR5 transgenes after injection into single cell embryos (zygotes) was determined. This was analyzed on day 3 after zygote microinjections.

For the first embryonic test, 11 embryos were injected using the 3kb Promoter/STOP/DR5 Tg DNA. A control Tg DNA that has no embryonic lethality and was previously found to produce high yield of viable founder Tg lines was injected into the same amount of embryos in the same condition. The development of embryos *in vitro* was evaluated after 3 days of Tg injection. Among the 11 DR5 injected embryos, 1 exhibited abnormal development, 2 developed to 1 cell, 5 developed to 2 cells, and 3 developed to morulae (>8 cells). None of them developed to blastocysts (~120 cells) (Fig 17). Among the 11 embryos that were injected with the control Tg DNA, 2 did not survive, 2 developed to 2 cells, 2 developed to 5 cells, and 5 developed to blastocysts (Fig 17). This first trial suggested that the present DR5 Tg may have induced developmental blocks to the embryos.

To avoid possible embryonic lethality and to enable better induction of arthritis and autoimmune disease, a construct with a full length c-FLIP_L within LoxP sites and upstream of the STOP sequence (Fig 18) was generated. This new construct is called 3 kb Pro-*Floxed* FLIP-STOP-h/m DR5. Figure 19 shows the 3 kb promoter/c-FLIP/STOP/chimeric DR5 graphic map.

FLIP (FLICE inhibitory protein) was originally identified in gammaherpesviruses and molluscipoxviruses (v-FLIP). It resembles caspase 8 in containing two DED domains but lacks the enzymatic C-terminal portion thereby competing with the recruitment of caspase 8 to FADD (Thome et al., Nature 386:517-21 (1997)). A mammalian cellular homologue (c-FLIP) has also been described, which exists in at least two splice variants, c-FLIP_L and c-FLIP_S (Irmeler et al., Nature 388:190-5 (1997)). The full-length c-FLIP_L shows overall structure homology to caspase 8 but bears a mutation in the caspase-like domain that renders it enzymatically inactive and thus inhibits death domain-induced cell death.

The impact of the 3 kb Pro- *Floxed* FLIP-STOP-h/m DR5 construct on embryonic development was studied. The same control construct tested above was used for comparison. Ten embryos were tested for each group. Microscopic observation of embryonic development was carried out 2 days after the injection. The results indicated that among the ten 3 kb Pro-*Floxed* FLIP-STOP-h/m DR5 construct

injected embryos, 1 was dead, 2 exhibited fragmented development, 1 developed to 1 cell, 0 developed to 2 cells, and 6 developed to morulae (>120 cells) (Fig. 20).

Among the 10 embryos that were injected with the control Tg DNA, 0 were dead, 4 exhibited abnormal fragmented development, 1 developed to 1 cell, 1 developed to 2 cells, and 4 developed to morulae (>120 cells) (Fig. 20). Because there was equivalent development of the 3 kb Pro-*Floxed* FLIP-STOP-h/m DR5 and the control construct injected embryos, this *in vitro* trial shows that the new anti-apoptosis FLIP_L inserted construct may rescue Tg mice from embryonic lethality caused by leaky hu/mo DR5 expression. Integration of transgenic DNA in the pre-implantation embryos was determined by nested PCR. See Figure 21.

Example 8: Development of Mice Expressing Chimeric DR5.

Mice were produced using a human/mouse chimeric DR5 in which the *Floxed* stop had been placed upstream of the ATG start site of the human/mouse chimeric molecule. As described above, this *Floxed* STOP element produced in human/mouse DR5 chimeric constructs is driven by the 3 kb promoter, a 3 kb promoter and first intron, or the 3 kb promoter/first intron and the 3' UTR of mouse. Mice were produced with the FLIP_L gene embedded into the *Floxed* sequence. This helped ensure survival during embryonic development of the mouse. This FLIP_L and the STOP sequence was removed by targeted expression of Cre. Figure 22 shows the genotyping of five (5) 3 kb/FLIP/STOP/Chimeric DR5 mice. Figure 23 shows the genotyping of thirteen (13) 3 kb/FLIP/STOP/Chimeric DR5 mice. Primers were targeted to the human portion of the chimeric DR5 polypeptide.

Example 9: Development of cIAP2 Construct.

An alternative construct with cIAP2 within the LoxP sites and upstream of the STOP sequence (called 3 kb Pro-*Floxed* IAP-STOP- h/m DR5) is generated. Members of a family of inhibitors of apoptosis (IAP), first described in insect viruses, are characterized by the presence of various number of Baculoviral IAP repeat (BIR) motifs. One of the important functions of IAPs is inhibition of apoptosis by binding to caspases and other BIR-interacting proteins. This new construct is used for development of other chimeric DR5 Tg lines of mice to enable study of the effects of DR5 antibodies in mice that develop autoimmune disease due to a decrease in the threshold of autoimmune disease induction by the expression of IAP.

Example 10: A Mouse Model of Arthritis that Expresses Chimeric DR5.

The DBA/1 collagen mouse model of arthritis is used. The DBA/1 (H2q) and B6 (H2b) mouse is used for studies of the effects of IL-17 on type II collagen (CII)-induced arthritis (CIA). B6 mice are relatively resistant to CIA compared to DBA/1 mice. However, cyropreserved H2q congenic B6 mice are available from the Jackson Laboratory (Stock Number: 000945). These mice are first acquired and used to backcross with the hu/mo chimeric DR5 Tg B6 mice (H2q hu/mo chimeric DR5 Tg mice) to induce CIA. A second backcross strain is also produced in which the DBA/1 mouse model is crossed back to the DR5 Tg mice using a speed-congenic approach with 50 markers to select for the DBA/1 genotype. The DR5 transgene and the DBA/1 MHC (DBA/1-hDR5 human/mouse chimeric Tg mouse) are selected. The DBA/1 H2q MHC is the most important locus in conferring susceptibility to arthritis in the DBA/1 mouse.

The H2q-hu/mo chimeric DR5 Tg mouse or the DBA/1-hDR5 human/mouse chimeric Tg mouse are used to induce CIA. CII arthritis is induced by injection of CII and complete Freund's adjuvant (CFA) in 3 to 4 wk-old mice following by a second injection of CII and CFA 3 weeks later. Arthritis is observed 10 to 14 days after the boost. To help ensure the development of arthritis and accelerate development of this mouse model, some mice are administered Ad-IL-17 before the priming dose of CII and CFA to accelerate the development of arthritis in strains of mice that are not normally susceptible to arthritis (including the C57BL/6 mouse that has the resistant H2b MHC allele).

Example 11: Chimeric Mouse Model for Optimization of DR5 Antibody for Cancer Therapy.

The DR5 chimeric Tg mouse is also used in combination with mouse tumor models. Studies are carried out by crossing the DR5 Chimeric 1 Tg mouse with an appropriate mouse model of cancer from Jackson Laboratories. The studies enable study of the effect of DR5 agonist agents, for example, an anti-human DR5 antibody, in mouse models of cancer.

Numerous mouse models of cancer are available commercially, from the Jackson Laboratories, and non-commercially. These mice develop cancer due to defects in regulation of cell growth or growth factors, proliferation, DNA repair, or

apoptosis. Mice are available with preferential development of adenomas of the lung, intestine, gonads or multiple tissues. Models of liver cancer have been developed. There are a large number of mouse models of breast cancer due to altered expression of genes under the control of the mouse mammary tumor virus (MMTV) promoter.

5 The selected mouse model of cancer is crossed with the hu/mo DR5 chimeric Tg mouse for studies of TRA-8 in treatment of established local or metastatic cancer.

By way of example, the FVB.Cg-Tg(MMTV-vHaras)SH1Lcd/J mouse (“MMTV-HaRas mouse”) is a mouse model of breast cancer maintained and available from the Jackson Laboratory. These transgenic mice express the Harvey RAS (human) gene under the direction of the mouse mammary tumor virus promoter. The majority of expression of the transgene is detected in the mammary tissue and salivary glands. Both male and female transgenic mice develop malignant lymphoid tissue and mammary and salivary gland tumors as early as 5 weeks of age. Half of the female transgenic animals develop tumors by 6 months of age. Histological analysis of mammary tumors reveal most of the tumors were adenocarcinomas and are locally invasive with some cases of metastasis to liver and/or lung. This MMTV-HaRas oncogene develops an oncogenic phenotype on a mixed B6/FVB mouse background. The MMTV-HaRas mouse is crossed with the chimeric DR5 Tg mouse to produce double Tg mice for studies of the effect of DR5 antibodies on mouse cancer development *in vivo*.

Example 12: Identification of anti-DR5 antibody as an anti-macrophage reagent for rheumatoid arthritis in a chimeric human/mouse DR5 mouse model.

Methods

Cell lines, cell preparation and culture. Mouse NIH3T3 and human HEK293 cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured in DMEM (Invitrogen; Carlsbad, CA) supplemented with 10% FBS, 100 units/ml penicillin, 100µg/ml streptomycin (Invitrogen), and 2mM glutamine (Invitrogen) at 37°C, 5% CO₂ in a humidified incubator. Single cell suspensions from spleen and inguinal lymph nodes were prepared as described before and cultured in RPMI-1640 (Invitrogen) supplemented with 10% FBS, 10mM HEPES and 0.1% 2-mercaptoethanol (Invitrogen).

Expression constructs, transfection, Western blot and ATP^{Lite}TM

analysis. Human and mouse DR5 cDNAs were obtained from Open Biosystems (Huntsville, AL). The extracellular domain of human DR5 was amplified by PCR using primer A 5'-actgtcgacgcccccaagtcagcctggacacata-3' (SEQ ID NO:24) and primer B 5'-tcctatccagaggcctagcttatgccaagaacagggagaggcaggagtcctgg-3' (SEQ ID NO:25). Similarly, the transmembrane and intracellular domains of mouse DR5 were amplified by PCR using primer C 5'-ccagggactcctgcctctccctgttcttggcataagctaggcctctggatagga-3' (SEQ ID NO:26) and primer D 5'-gatgcggccgctcaaacgcactgagatcctcctgg-3' (SEQ ID NO:27). There are 49 base pairs overlapping between these two PCR products. The fused chimeric DR5 was then generated by PCR using the mixture of above 2 products as the template and primers A and D. The purified PCR product was then digested by *SaII* and *NotI* and this *SaII-NotI* chimeric DR5 fragment was used to replace the human DR5 on the pCMV-SPORT6 vector. A 3 kb putative mouse DR5 promoter was cloned from mouse BAC RP24-355K8. This regulatory genomic DNA sequence was then subcloned upstream of the chimeric DR5. All constructs were sequenced to confirm their identities.

Transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Forty-eight hours after transfection, cells were lysed and a 50 µg sample of the protein lysate was analyzed by Western blot as described. Cell viability was determined by an ATP luminescence assay kit (PerkinElmer; Waltham, MA) following the manufacturer's instructions.

Establishment of the hu/mo chimeric DR5 Tg mouse. The DNA used for generating DR5 Tg mice was based on the 3 kb mouse promoter/Chimeric DR5 construct described above. To further enable the tissue specific expression of chimeric DR5, a *Floxed-STOP* cassette (Addgene; Cambridge, MA) was introduced in between the promoter and chimeric DR5. All constructs were sequenced to confirm their identities. A purified 8.3 kb *DrdI-DrdI* fragment was then used to generate the Tg mice by single cell embryo microinjection. Genotyping was performed by using the primers that target the human DR5 extracellular domain.

Generation of chimeric DR5 Ubc Cre double Tg and chimeric DR5 Lyz.Cre double Tg mice. For allowing the expression of chimeric DR5, the *Floxed-STOP*-chimeric DR5 Tg mice were bred with two different Cre-expressing mice: (1)

the UBC-cre/ESR1)1Ejb/J (The Jackson Laboratory; Bar Harbor, ME), which has strong tamoxifen-inducible *cre* activity in all reported tissues, and (2) Lysozyme M Cre (B6.129-Lyzs^{tm1(cre)Ifo}/J (The Jackson Laboratory), which expresses Cre in myeloid cells.

5 **Quantitative reverse transcription PCR (qRT-PCR).** Total RNA was isolated from different mouse tissues using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. RNA concentration was assessed by spectrophotometry. The ratios of 260nm/280nm absorbency were above 1.95. The RNA quality was also verified by Bio-Rad Experion system (Bio Rad Laboratories, Inc.; Hercules, CA). Two micrograms of total RNA was used to synthesize the first-
10 strand cDNA by using random hexamer primers and RevertAidTMM-MuLV Reverse Transcriptase (Fermentas Life Science; Glen Burnie, MD). QRT-PCR was performed on an IQ5 multicolor real-time PCR detection system). The PCR reaction was done in 25µl solution containing 12.5µl SYBR Green Supermix (Bio-Rad), 1µl cDNA and
15 400nM of each primer, with the following setting: 95°C for 3 minutes followed by 40 cycles of 95°C for 10 seconds and 60°C for 30 seconds. Quantification of gene expression was analyzed using the IQ5 Optical System Software version 2.0.

Flow cytometric analysis.

Induction of collagen II-induced arthritis (CIA). CIA was induced using
20 DR5 Tg mice that were 8-16 weeks old on the C57BL/6 background. Mice were immunized by intradermal administration of chicken type II collagen (Chondrex Inc.; Redman, WA) emulsified in Complete Freund's Adjuvant (CFA) followed by a boost of CII in Incomplete Freund's Adjuvant (IFA) on day 30 after the primary injection as previously described. To ensure more complete and uniform induction of CII arthritis
25 with a higher incidence, an adenovirus expressing mouse IL-17 (2x10⁹ pfu / mouse) was administered to all mice 2 days prior to the CII primary immunization. The severity of arthritis was measured daily based on the 0-3 scale system as follows: 0, normal; 1, erythema and mild swelling confined to the ankle, tarsal or one digit; 2, erythema and moderate swelling extending from the ankle to tarsal, metatarsal or two
30 or more digits; 3, erythema and severe swelling encompassing the ankle, foot and digits. The maximal clinical score for each mouse was 12.

TRA-8 treatment to CIA mice. TRA-8 (0.2 mg per mouse) or IgG1 isotype control was administrated intravenously (iv) every 3-7 days starting on day 0 (early treatment) or on day 30 (late treatment) until mice were sacrificed.

Histopathological assessment and immunohistochemical staining. Limbs including knee joints, ankle joints and paws were removed and fixed 4% formaldehyde for 72 hours and then decalcified in fresh prepared 10% EDTA in 1 X PBS, which was changed every other days for 2 weeks. The joints were then embedded in paraffin. Tissue sections of 5 μ m were stained with hematoxylin and eosin and examined by light microscopy. A histological analysis of synovium, bone and cartilage was performed, which includes an evaluation of inflammation cell infiltration, synovial hyperplasia, pannus tissue formation, cartilage destruction, and cortical bone erosion. Brain, lung, liver, kidney and other tissues were also assessed for morphological changes. TUNEL staining for apoptosis of synovial cells was carried out as previously described. Immunohistochemical staining using anti-Mac-3 (clone M3/84, BD Biosciences; San Jose, CA) as a macrophage marker was performed as described previously.

***In vivo* imaging of arthritis using ProSense 750, a protease-activated near-infrared fluorescence (NIRF) probe.** Mice were injected intravenously with 2 nmol ProSense 750TM (ViSen; Bedford, MA) in 150 μ l PBS. Twenty-four hours after injection, mice were anaesthetized with Nembutal (Ovation Pharmaceuticals) and imaged using the Odyssey Infrared imaging System (LI-COR, Lincoln, Nebraska).

Human rheumatoid arthritis (RA) synovial fragment and fluid studies.

Fresh synovial fluid from RA patients ($n=15$) was obtained at their clinical visiting. All subjects fulfilled the American College of Rheumatology criteria for RA. Synovial fragments were isolated by low speed centrifugation and mononuclear cells were isolated by using Ficoll-paque solution (Amersham Biosciences; Piscataway, NJ) following the manufacturer's manual. Fresh synovial fragments were treated with and without TRA-8 (2 μ g/ml for 16 hours) followed by staining with 7-AAD (Enzo Life Science; Plymouth Meeting, PA). Synovial fragments fixed with 4% formaldehyde were stained by TRA-8 (5 μ g/ml) following by goat anti-mouse IgG conjugated with Alexa 488 (Invitrogen), anti-human CD68 (BioLegend; San Diego, CA), and anti-human CD90 (BioLegend) conjugated with Alexa 555. Fragments

were counterstained with Hoechst 33258 (Invitrogen). Synovial fragments and cells were observed using the LSM 710 confocal microscopy (Zeiss; Thornwood, NY). FACS analysis was carried out as described above.

Statistics. Figures are representative of at least 3 independent experiments.

5 Statistical analyses were performed using 2-tailed Student's *t* test. *P* values less than 0.05 were considered statistically significant.

Results

Cellular antagonist reagents such as anti-CD20 and anti-CD64 that eliminate autoimmune pathogenic cells often act via an antibody- or complement-mediated pathway to induce cytolysis. In contrast, successful anti-DR5-mediated apoptosis to eliminate pathogenic cells in a mouse model of RA requires not only expression of human DR5 on the surface of cells but also proper docking and activation of mouse death domain adaptor molecules to activate the apoptosis cascade. To accomplish this, a chimeric human/mouse DR5 molecule was generated that can confer TRA-8 mediated DR5 apoptosis. The expression and function of human DR5, mouse MK, which is the mouse homolog of human DR5 (Wu et al., Cancer Res. 59:2770-5 (1999)), and the hu/mo DR5 chimeric molecule consisting of the extracellular domain (ED) of human DR5, the transmembrane (TM) and intracellular domain (ID) of mouse MK were compared (Fig. 24). The cell surface expression of human DR5 and chimeric DR5 was comparable whereas the 3 kb *MK* promoter exhibited slightly lower activity compared to a CMV promoter (Fig. 24C).

The apoptotic susceptibility to TRA-8 was next determined in human HEK293 cells and mouse NIH3T3 cells expressing the human DR5, the mouse DR5, and the chimeric DR5 driven by the CMV promoter. HEK293 cells that were transfected with human and chimeric DR5 exhibited approximately 60% reduction of viability by TRA-8 treatment. HEK293 cells transfected with mouse DR5 did not exhibit a significant killing effect by TRA-8 (Fig. 25A). These suggested that extracellular and death domain of human and mouse DR5 function properly in human and mouse cells, but the mouse death domain was silent in human cells. The same constructs were transfected into mouse NIH3T3 cells and only the chimeric DR5 exhibited proper killing effects prompted by TRA-8 (Fig. 25B). This indicated that the human death

domain was not functioning in mouse cells. Therefore the present approach using a chimeric human ED and mouse TM/ID is necessary and feasible for analysis of TRA-8-induced apoptosis in mouse models of diseases. Chimeric DR5 Tg mice were generated using the chimeric DR5 driven by the mouse *MK* 3 kb putative promoter.

To avoid potential developmental defects of either the embryo or the immune system, a *Floxed-STOP* sequence was introduced between the promoter and the chimeric DR5 (Fig. 25C). The 3 kb promoter plays the major regulation role after the *Floxed-STOP* was removed. After induction of Cre by TMX, various levels of chimeric *DR5* Tg transcript can be detected in the examined tissues (Fig. 25D) and its expression pattern mimics that of the endogenous mouse *MK*.

The DR5 Ubc.Cre double Tg mice and the Ubc.Cre single Tg mice were induced to develop CIA. There was significant development of early joint swelling and erythema response (from day 20 to day 31 after the primary CII injection, peak on day 26) in all the experimental groups except the chimeric DR5 Ubc.Cre double Tg treated with early TRA-8 in which the development of the joint swelling response was significantly decreased (Fig. 26A, closed circle). In addition, late TRA-8 treatment initiated 2 days before the CII boost also significantly inhibited the late phase of CIA after CII boost (Fig. 26A, open circle). In contrast, all chimeric DR5 negative Tg mice (with and without TRA-8 treatment) and double Tg mice without TRA-8 treatment developed significant arthritis after both the primary and the secondary CII injection. Joint histopathological studies showed a decreased severity of inflammatory cell infiltration, synovial hyperplasia, cartilage damage, and bone erosion after TRA-8 treatment in chimeric DR5 Tg mice (Fig. 26B, left panels). Immunohistochemistry staining further demonstrated a dramatic reduction of Mac-3⁺ macrophages in the synovium by TRA-8 treatment (Fig. 26B, right panel). During the acute phase of CII arthritis, chimeric DR5 is expressed in CD11b⁺Gr-1⁺ granulocytes, and CD11b⁺Ly6C⁺ inflammatory macrophages (Figs. 26C and 26D). There was a lower expression of chimeric DR5 on CD4⁺ T cells, CD8⁺ T cells and minimal expression on CD19⁺ B cells (Figs. 26C and 26D). TRA-8 treatment reduced the CD11b^{high} activated macrophages by ~50-60% (Figs. 26E and 26F). Thus, the activation status of the cells appears critical for the TRA-8 mediated apoptosis.

Chimeric DR5 is highly expressed in CD11b⁺ macrophages in the Tg mice. To further determine if TRA-8 can specifically inhibit macrophage proliferation, a single cell suspension was prepared from draining inguinal lymph nodes (LN) from CII-induced DR5 transgenic mice and stimulated with LPS (5 µg/ml). [³H]-thymidine incorporation was analyzed on Days 2 and 5. On Day 2, there was comparable [³H]-thymidine incorporation by LPS stimulated cells in the draining LN from both Ubc.Cre single Tg or DR5 Ubc.Cre double Tg mice (Fig. 26). There was no difference in LPS stimulated [³H]-thymidine uptake in draining LN in treatment with TRA-8 in DR5 Ubc.Cre double Tg mice compared to Ubc.Cre single Tg mice. However, on Day 5 after treatment with LPS, there was a significant decrease of thymidine incorporation by macrophages from DR5 Ubc.Cre double Tg mice by *in vitro* TRA-8 treatment, which was not found in Ubc.Cre single Tg mice (Fig. 26). These results suggest that TRA-8 can inhibit proliferation of macrophages stimulated by LPS and most likely by induction of apoptosis in these cells. This occurs on Day 5 and is most significant at this late time point. This is important since draining LN macrophages are thought to play a stronger role in the antigen specific T cell responses that are induced by CII the boost.

Because of the essential roles of macrophages in inducing and perpetuating both synovial inflammation and bone erosion in arthritis, the targeted depletion of macrophages with TRA-8 was tested. To achieve restricted expression of the chimeric DR5 on myeloid lineage cells, the *Floxed-STOP* chimeric DR5 Tg mice were crossed with the Lysozyme (Lyz).Cre mice in which Cre is driven by the Lyz promoter. This resulted in specific expression of chimeric DR5 on ~23% of the CD11b⁺ macrophages (Fig. 27A). Furthermore, *in vivo* TRA-8 treatment (0.2 mg iv, every 3 days x 3) reduced the CD11b⁺ macrophages from ~2% to ~0.8% (Fig. 27B). Together, these results indicate that chimeric DR5 is expressed in CD11b⁺ macrophages in the spleen of DR5 Lyz.Cre double-Tg mouse, and that CD11b⁺ macrophages can be depleted by >50% *in vivo* by administration of TRA-8. It was next determined if TRA-8 exhibited similar therapeutic effects in Lyz.Cre DR5 mice as found in Ubc.Cre DR5 mice. CIA was induced in DR5 Lyz.Cre mice. Early arthritis was first seen in Lyz.Cre mice that were not treated with TRA-8 starting at day 15 after primary CII injection. By day 23, arthritis became apparent in all four

groups of mice. After initiation of TRA-8 treatment (0.2mg/mouse iv every 3 to 7 days) on day 28 and CII boost on day 30, Lyz.Cre mice with and without TRA-8 treatment and Lyz.Cre DR5 Tg mice without TRA-8 treatment developed arthritis further. However, the Lyz.Cre DR5 Tg mice treated with TRA-8 exhibited a significantly lower arthritis severity (Fig. 27C). These data demonstrated that targeted depletion of macrophages by TRA-8 can ameliorate CIA.

Cathepsin B is predominately produced by macrophages associated with the severity of arthritis. To determine if TRA-8 treatment in Lyz.Cre DR5 Tg mice can reduce cathepsin B activity, CIA was induced in DR5 Tg and control mice treated with and without TRA-8. Twenty-four hours after ProSense 750TM injection, mice were imaged using the Odyssey Infrared imaging System. Notably, increased activated cathepsin B was found in the Lyz.Cre single Tg mouse treated with and without TRA-8 and Lyz.Cre DR5 Tg mice treated with TRA-8. The increased protease activity was apparent in the ankle, tarsal joints and digits. Only minimal protease activity was found in the representative TRA-8 treated DR5 Lyz.Cre double-Tg mouse (Fig. 27D). The imaging results are highly correlated with the clinical scores. Because increased cathepsin activity is associated with macrophage activation and collagen degradation, these data further demonstrated the role of macrophages in the development of CIA and the therapeutic effects of depleting activated macrophages by TRA-8.

To demonstrate directly that TRA-8 can deplete macrophages in human RA synovium, fresh synovial fluid (SF) samples were obtained from 15 RA patients attending an outpatient rheumatology clinic. Synovial fragments predominantly from the synovial lining layers were isolated from the SF. Immunofluorescence staining using anti-CD90 and anti-CD68 was carried out for identification of fibroblasts and macrophages, respectively. The results indicated that RA synovial lining layer consists of 75% macrophages and 25% fibroblasts compared to 30% and 70% in OA synovium respectively (Fig. 28A). TRA-8 staining on the fixed synovial fragment indicated the high expression of DR5 on the surface of synovial tissue fragment macrophages (Fig. 28B). After incubation with TRA-8 (1 µg/ml) for 16 hrs, 7-AAD staining of the synovial fragments exhibited a dramatic induction of cell death compared to the no TRA-8 treated samples (Fig. 28C). Further studies using FACS

analysis and ATPLite™ assay suggested that ~50% of the macrophages from the synovial fluid were killed by TRA-8 (Figs. 28D and 28E). These observations were consistent with DR5 Tg mouse data and demonstrated that selective elimination of the human synovial macrophages by TRA-8 is feasible.

WHAT IS CLAIMED IS:

1. A transgenic animal comprising one or more cells expressing a chimeric DR5 polypeptide.
2. The transgenic animal of claim 1, wherein the chimeric DR5 polypeptide comprises (i) the extracellular domain of human DR5 and (ii) the intracellular domain of a non-human DR5.
3. The transgenic animal of claim 2, wherein the chimeric DR5 polypeptide further comprises a transmembrane domain of a non-human DR5.
4. The transgenic animal of claim 3, wherein the intracellular domain and the transmembrane domain are from the same or different species.
5. The transgenic animal of claim 2, wherein the extracellular domain comprises SEQ ID NO:15.
6. The transgenic animal of claim 2, wherein the intracellular domain comprises SEQ ID NO:17.
7. The transgenic animal of claim 3, wherein the transmembrane domain comprises SEQ ID NO:16.
8. The transgenic animal of claim 2, wherein the intracellular domain is the same species as the transgenic animal.
9. The transgenic animal of claim 8, wherein the species is selected from the group consisting of a rat, guinea pig, rabbit and non-human primate.
10. The transgenic animal of claim 1, wherein the chimeric DR5 polypeptide comprises SEQ ID NO:1, SEQ ID NO:2 or SEQ ID NO:3.
11. The transgenic animal of claim 1, wherein the animal is a mouse.
12. The transgenic animal of claim 1, wherein the cells are T-cells, B cells, macrophages or fibroblasts.
13. The transgenic animal of claim 1, wherein the cells are cancer cells.
14. The transgenic animal of claim 1, wherein the animal is a model for arthritis or autoimmune disease.
15. The transgenic animal of claim 1, wherein the animal is a model for cancer.
16. The transgenic animal of claim 1, wherein the genome of the cells comprises SEQ ID NO:14.

17. A nucleic acid sequence encoding a chimeric DR5 polypeptide, wherein the chimeric DR5 polypeptide comprises (i) the extracellular domain of human DR5 and (ii) the intracellular domain of non-human DR5.
18. The nucleic acid sequence of claim 17, further comprising the transmembrane domain of a non-human DR5.
19. The nucleic acid sequence of claim 17, wherein the non-human DR5 is mouse DR5.
20. The nucleic acid sequence of claim 18, wherein the non-human DR5 is mouse DR5.
21. The nucleic acid sequence of claim 17, wherein the extracellular domain of human DR5 comprises SEQ ID NO:15.
22. The nucleic acid sequence of claim 20, wherein the transmembrane domain of mouse DR5 comprises SEQ ID NO:16.
23. The nucleic acid sequence of claim 19, wherein the intracellular domain of mouse DR5 comprises SEQ ID NO:17.
24. The nucleic acid sequence of claim 17, wherein the chimeric DR5 polypeptide comprises SEQ ID NO:1, SEQ ID NO:2 or SEQ ID NO:3.
25. An expression vector comprising a first nucleic acid sequence, wherein the first nucleic acid sequence is the nucleic acid sequence of any one of claims 17-24.
26. The expression vector of claim 25, further comprising a promoter sequence operably linked to the first nucleic acid sequence.
27. The expression vector of claim 26, wherein the promoter sequence comprises SEQ ID NO:6, SEQ ID NO:7 or SEQ ID NO:8.
28. The expression vector of claim 25, further comprising a second nucleic acid sequence encoding the first intron of a mouse DR5 polypeptide.
29. The expression vector of claim 28, wherein the second nucleic acid sequence is located 3' to the promoter sequence and 5' to the first nucleic acid sequence.
30. The expression vector of claim 25, further comprising regulatory elements.
31. The expression vector of claim 30, wherein the regulatory element is a *Floxed-STOP* element.
32. The expression vector of claim 25, further comprising SEQ ID NO:18.
33. A cell comprising the expression vector of claim 25.

34. The cell of claim 33, wherein the cell is a cancer cell.
35. The cell of claim 33, wherein the cell is a T-cell, a B-cell, a macrophage or a fibroblast.
36. A cell comprising the nucleic acid sequence of any one of claims 17-24, wherein the nucleic acid sequence is located in the genome of the cell.
37. The cell of claim 36, wherein the nucleic acid sequence is operably linked to a promoter sequence.
38. The cell of claim 37, wherein the promoter sequence comprises SEQ ID NO:6, SEQ ID NO:7 or SEQ ID NO:8.
39. The cell of claim 36, wherein the cell is a cancer cell.
40. The cell of claim 36, wherein the cell is a T-cell, a B-cell, a macrophage or a fibroblast.
41. A chimeric DR5 polypeptide sequence, wherein the chimeric DR5 polypeptide sequence comprises (i) the extracellular domain of human DR5 and (ii) the intracellular domain of a non-human DR5.
42. The chimeric DR5 polypeptide sequence of claim 41, further comprising the transmembrane domain of a non-human DR5.
43. The chimeric DR5 polypeptide sequence of claim 41, wherein the non-human DR5 is mouse DR5.
44. The chimeric DR5 polypeptide sequence of claim 42, wherein the non-human DR5 is mouse DR5.
45. The chimeric DR5 polypeptide sequence of claim 41, wherein the extracellular domain of human DR5 comprises SEQ ID NO:15.
46. The chimeric DR5 polypeptide sequence of claim 44, wherein the transmembrane domain of mouse DR5 comprises SEQ ID NO:16.
47. The chimeric DR5 polypeptide sequence of claim 43, wherein the intracellular domain of mouse DR5 comprises SEQ ID NO:17.
48. The chimeric DR5 polypeptide sequence of claim 41, wherein the polypeptide sequence comprises SEQ ID NO:1, SEQ ID NO:2 or SEQ ID NO:3.
49. A cell comprising the chimeric DR5 polypeptide of any one of claims 41-48.

50. A method of making a cell expressing a chimeric DR5 polypeptide comprising transforming the cell with a vector comprising the nucleic acid sequence of SEQ ID NO:14 or the nucleic acid sequence of any one of claims 17-24.
51. The method of claim 50, wherein the vector is an expression vector.
52. The method of claim 50, wherein the vector is an integration vector.
53. A method of screening for an agent or combination of agents for DR5-mediated apoptosis activity comprising
- (a) providing the transgenic animal of any one of claims 1-16;
 - (b) administering to the animal the agent or agents to be tested; and
 - (c) determining whether the agent or agents cause apoptosis of the cells expressing the chimeric DR5 polypeptide, wherein apoptosis indicates the agent or agents have DR5-mediated apoptosis activity.
54. The method of claim 53, wherein the agent is an antibody.
55. The method of claim 53, wherein the agent is a small molecule.
56. A method of screening for an agent or combination of agents for DR5-mediated apoptosis activity comprising
- (a) providing the cells of claim 49;
 - (b) contacting the cells with the agent or agents to be tested; and
 - (c) determining whether the agent or agents cause apoptosis of the cells, wherein apoptosis indicates the agent or agents have DR5-mediated apoptosis activity.
57. The method of claim 56, wherein the agent is an antibody.
58. The method of claim 56, wherein the agent is a small molecule.

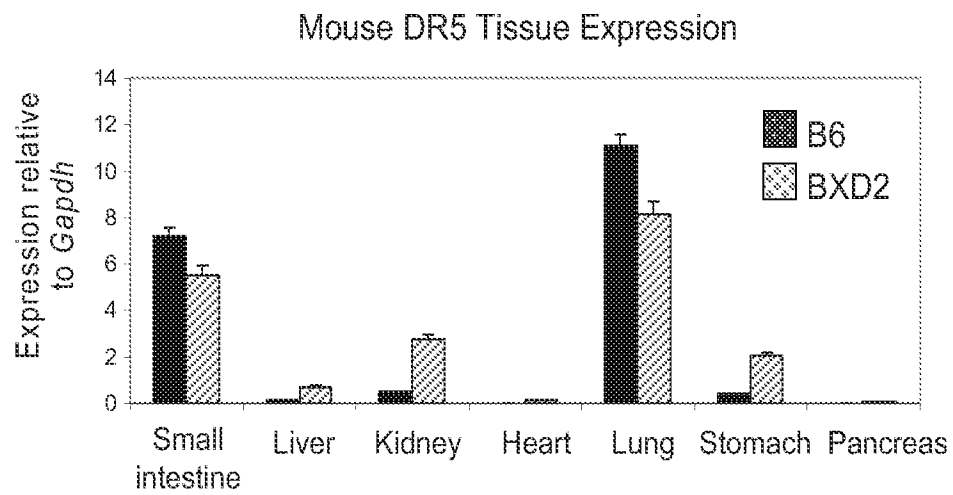


FIG. 1

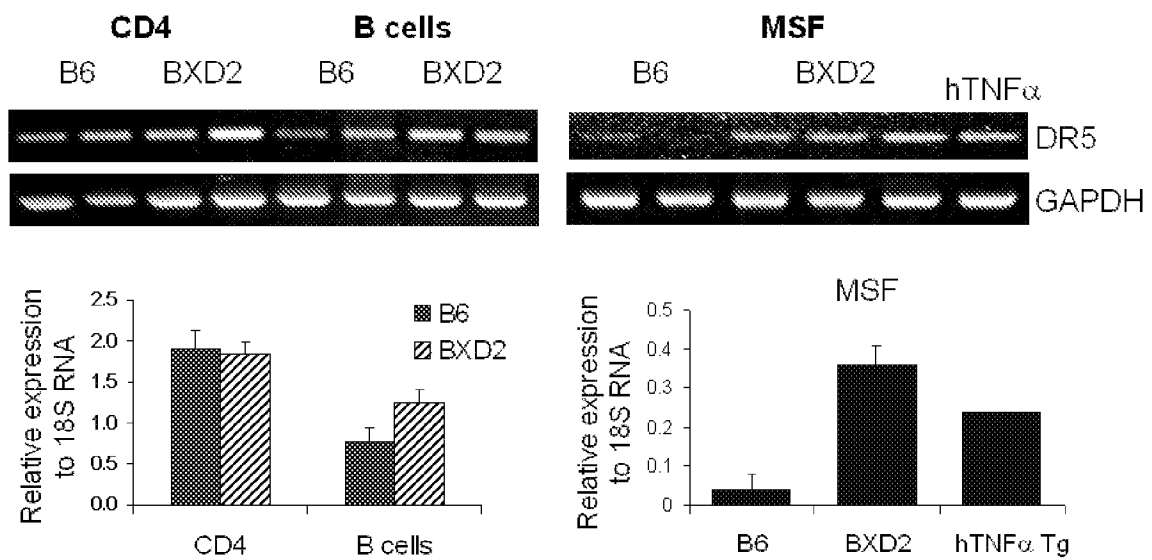


FIG. 2

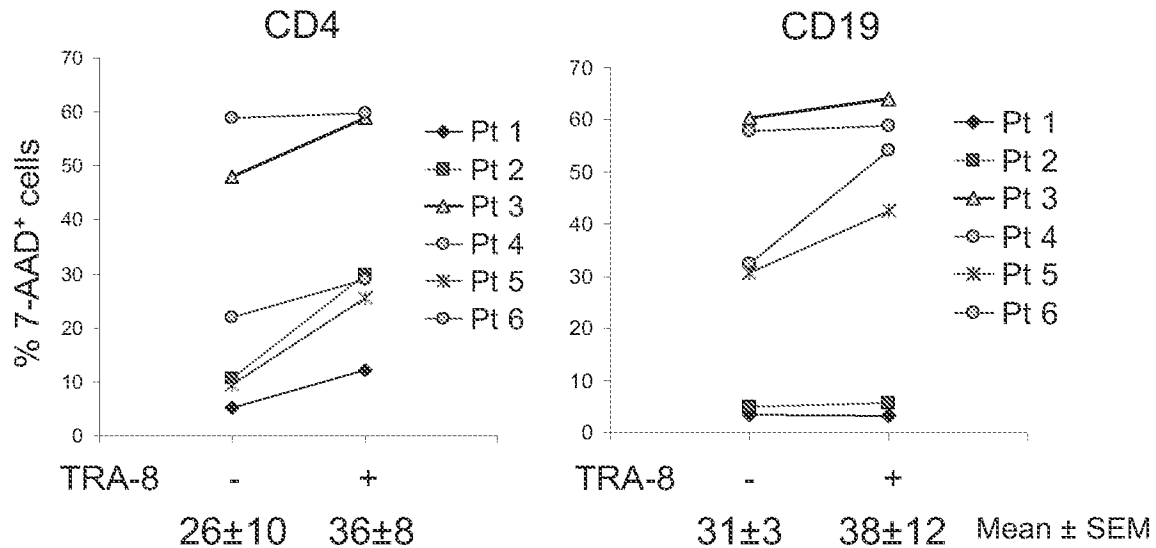
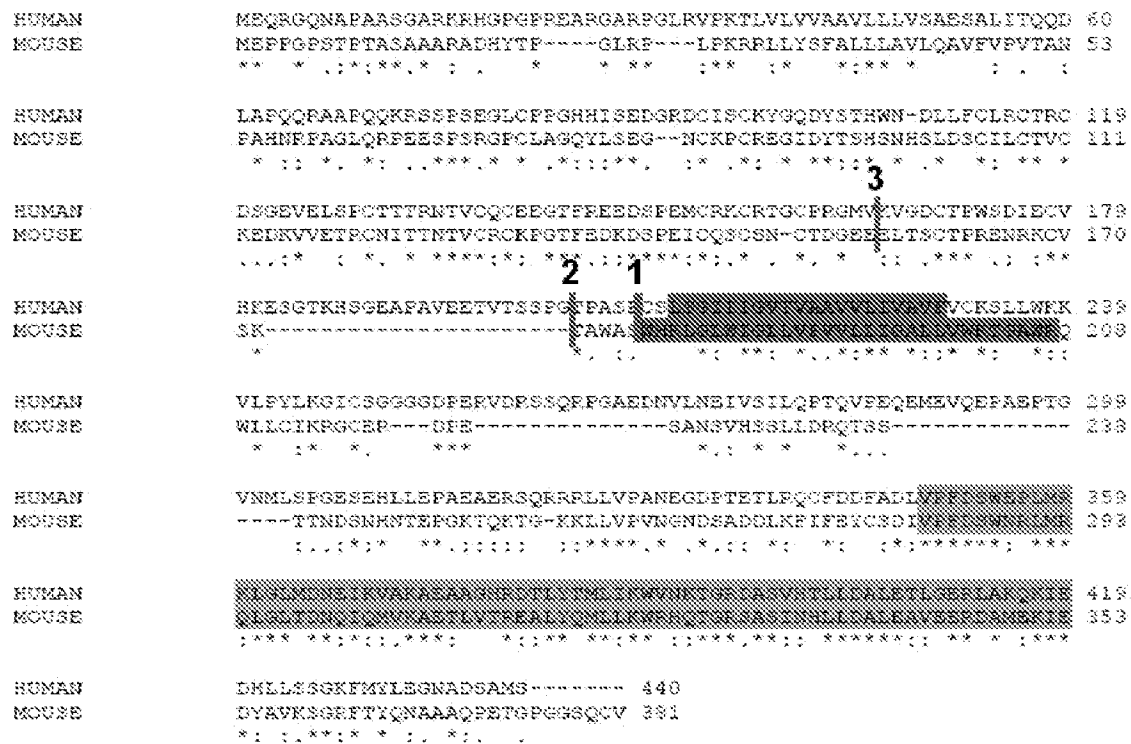


FIG. 3



 Transmembrane Domain
 Death Domain
 Human and Mouse DR5 Junction

FIG. 4

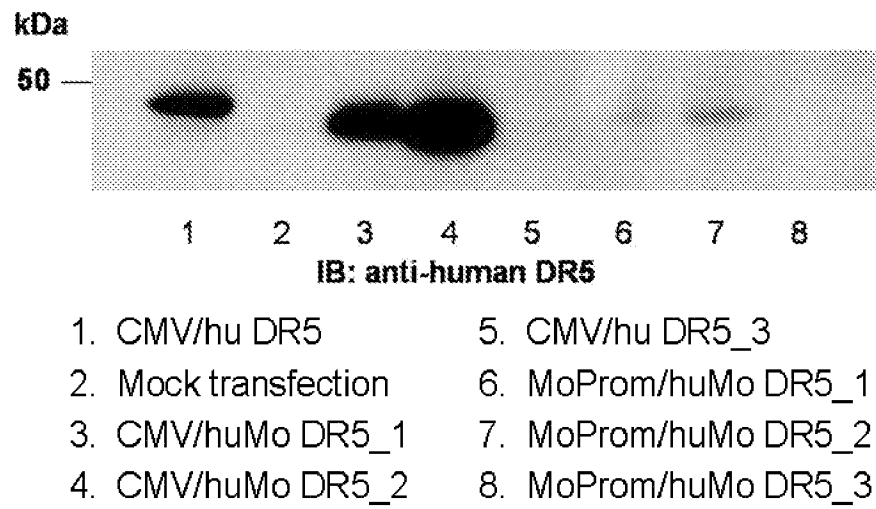


FIG. 5

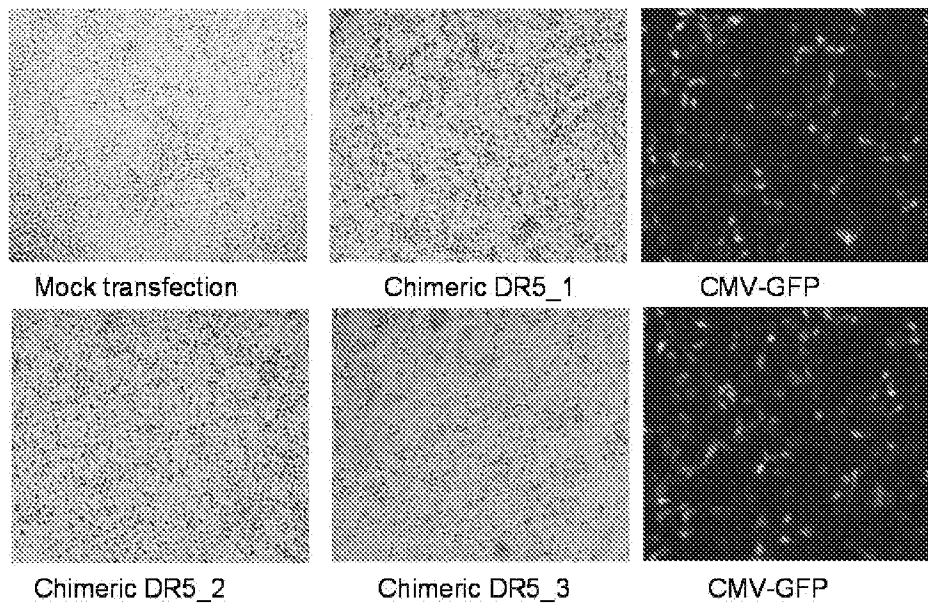


FIG. 6

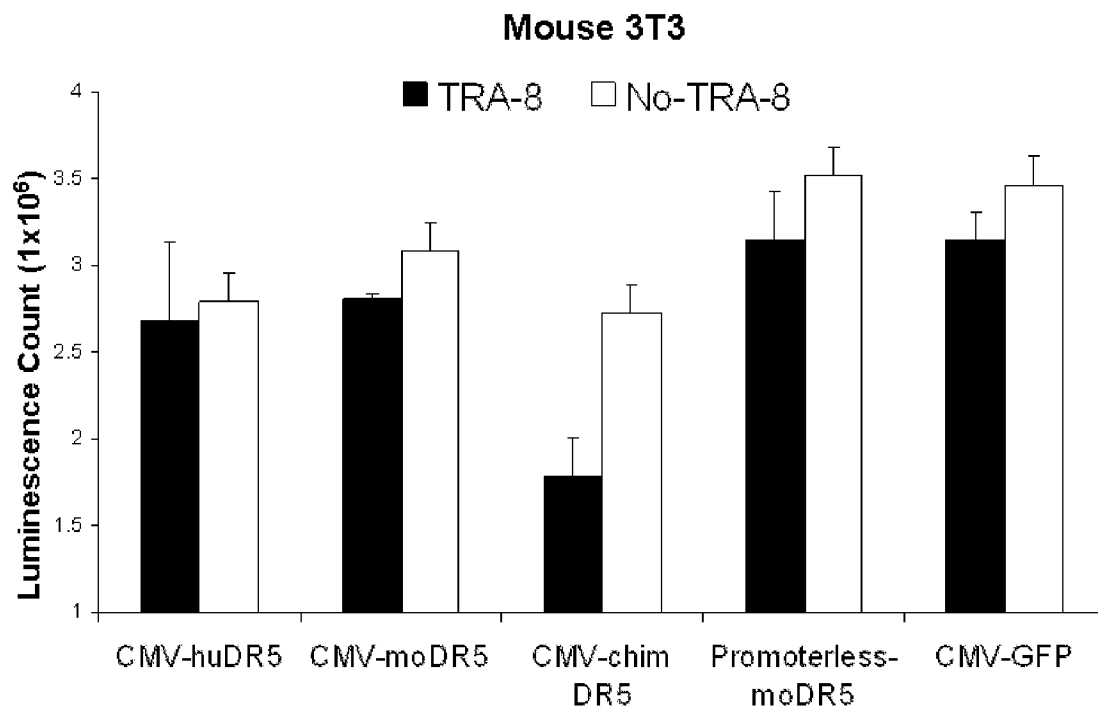


FIG. 7A

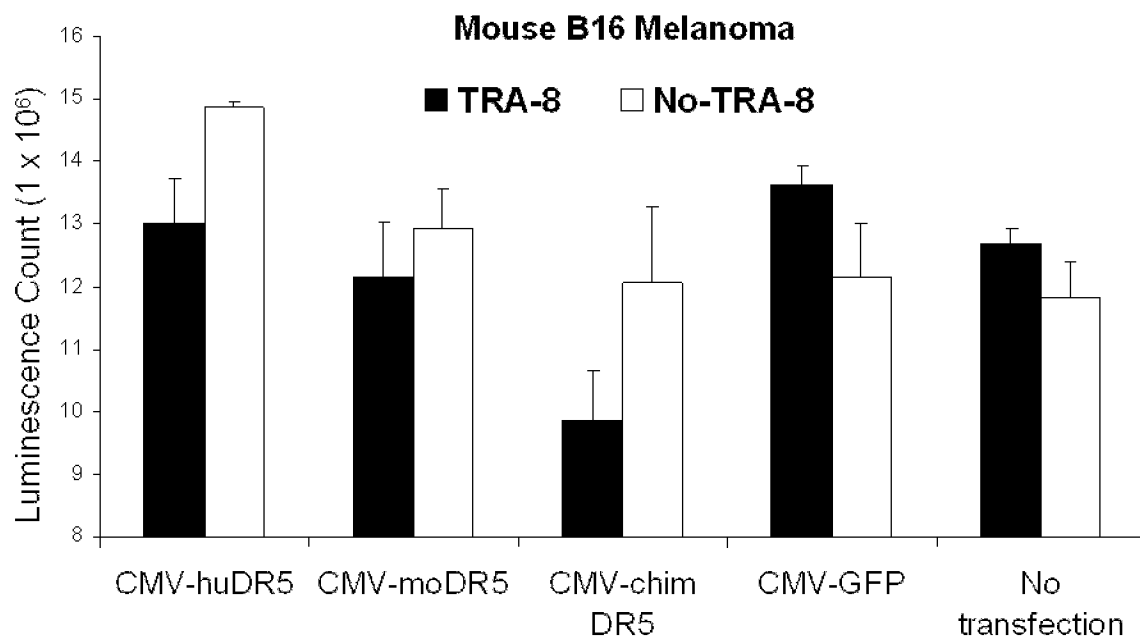


FIG. 7B

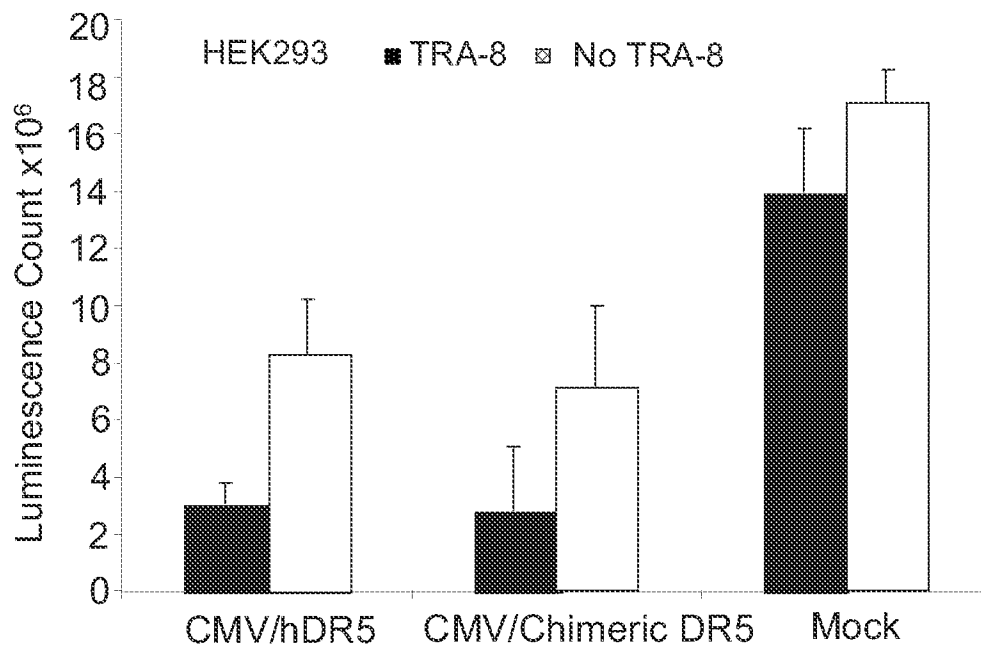


FIG. 8

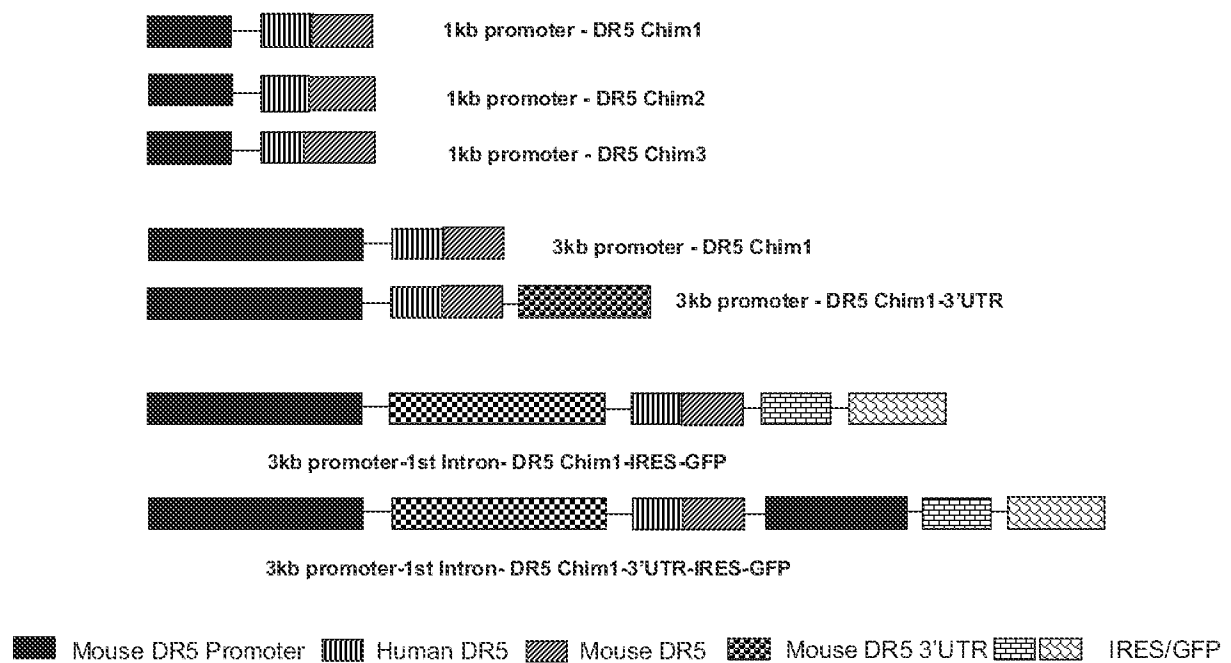
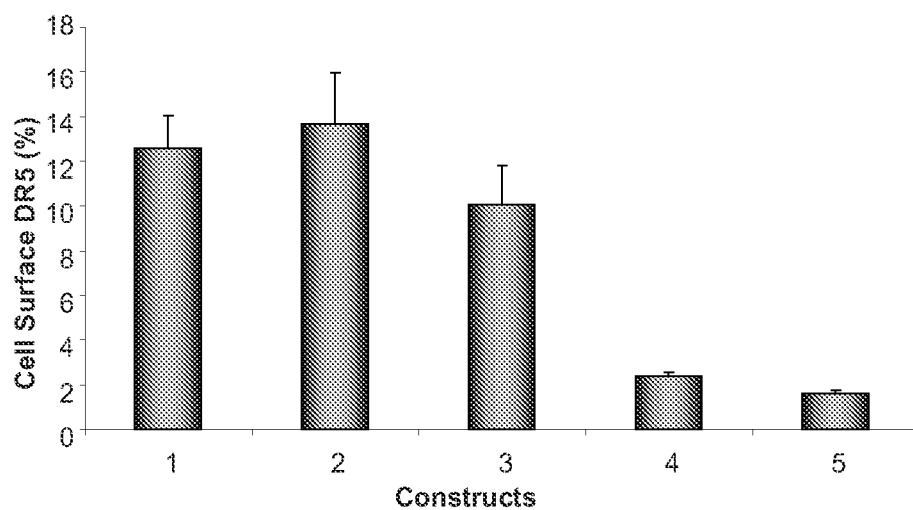


FIG. 9



1. CMV/DR5

2. 1kb promoter/DR5

3. 3kb promoter/DR5

4. CMV/DR5/IRES/GFP

5. 3kb promoter/1st intron/DR5/IRES/GFP

6. 3kb promoter/1st intron/DR5/3UTR /IRES/GFP

FIG. 10

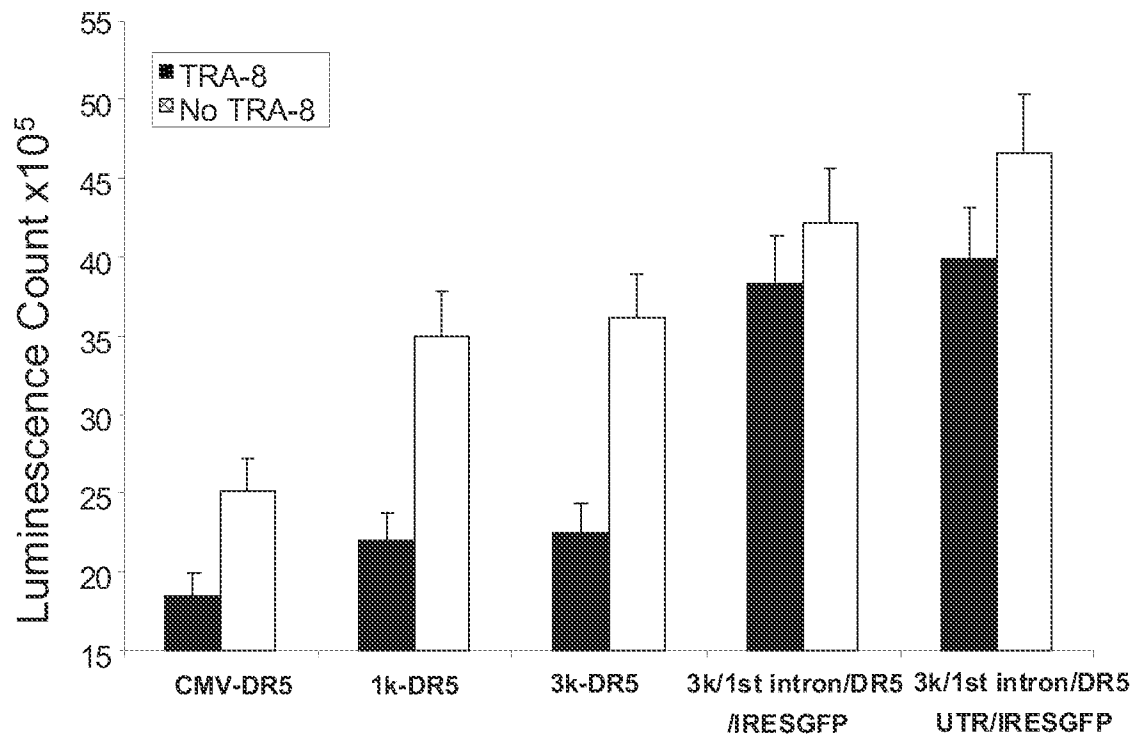


FIG. 11

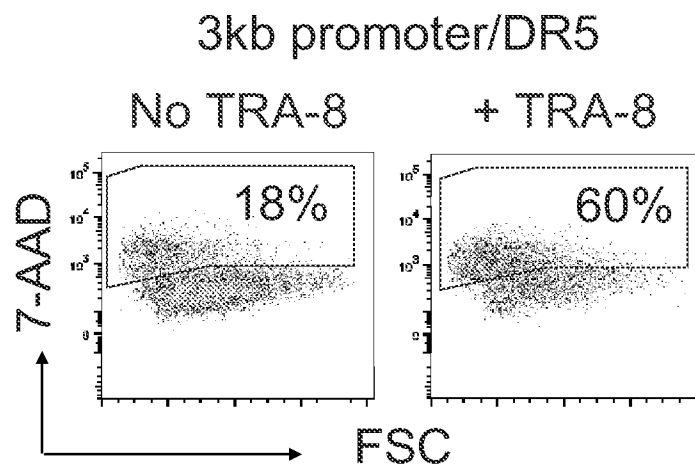


FIG. 12

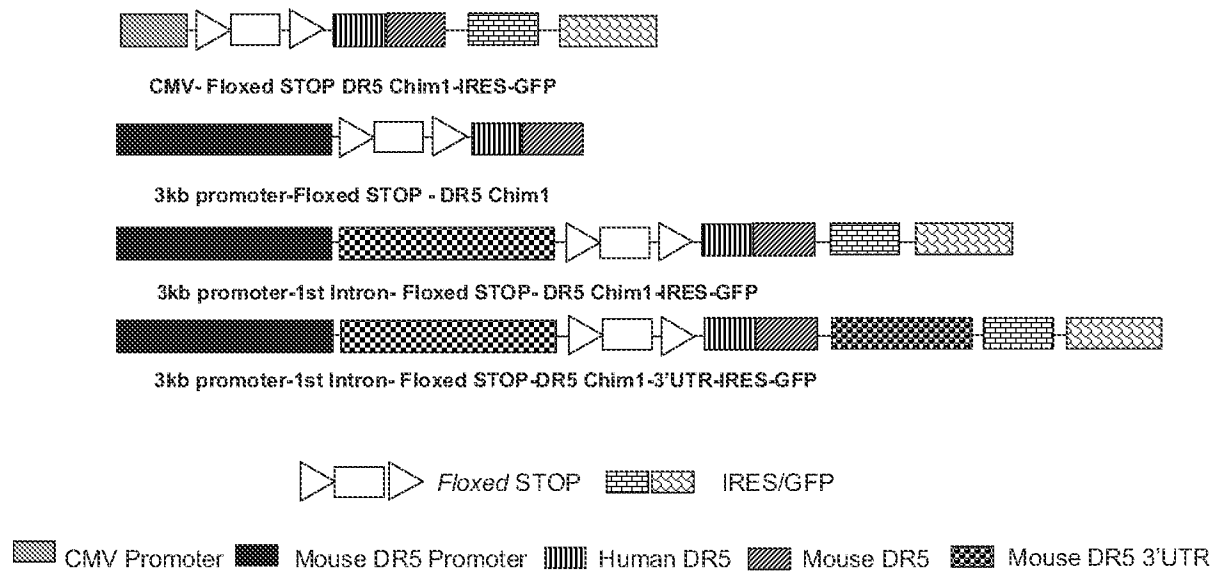


FIG. 13

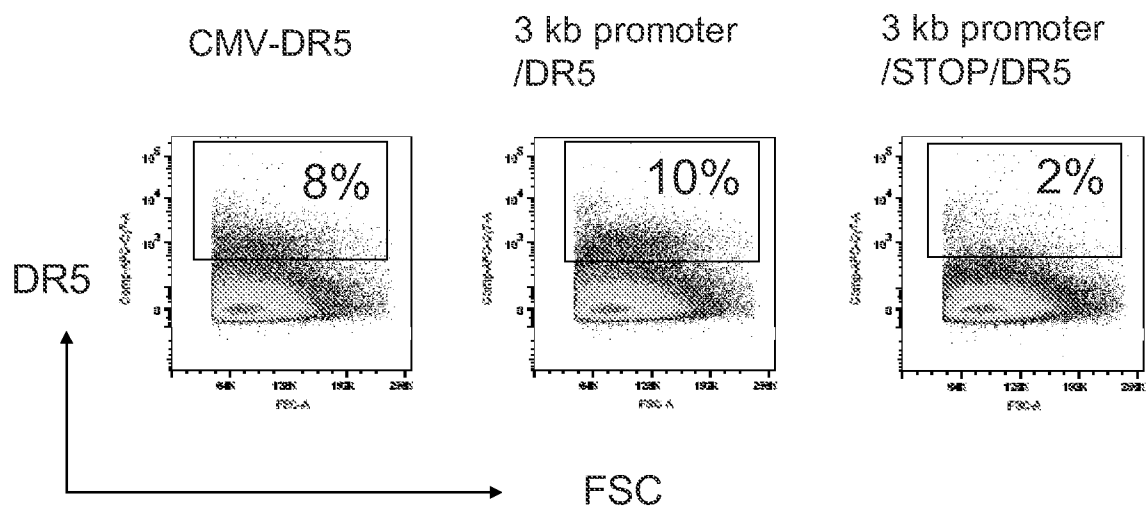


FIG. 14

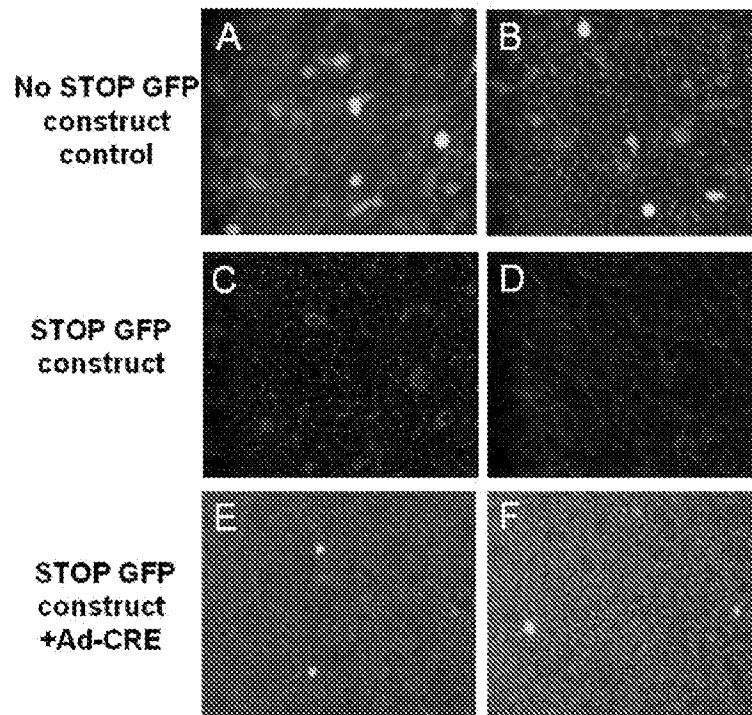


FIG. 15

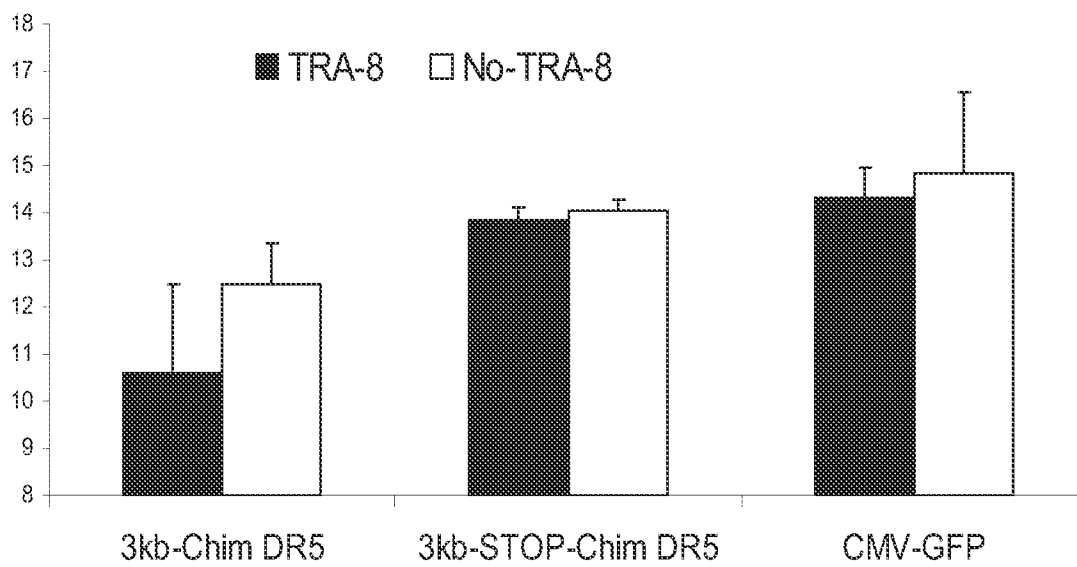


FIG. 16

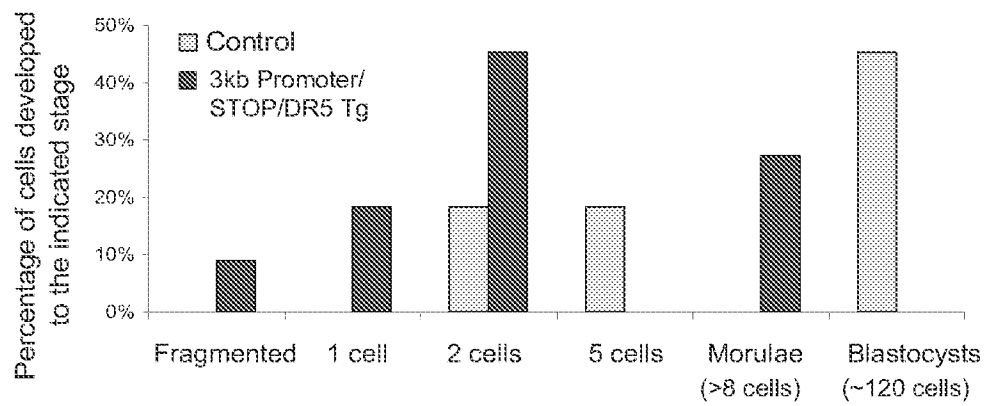


FIG. 17

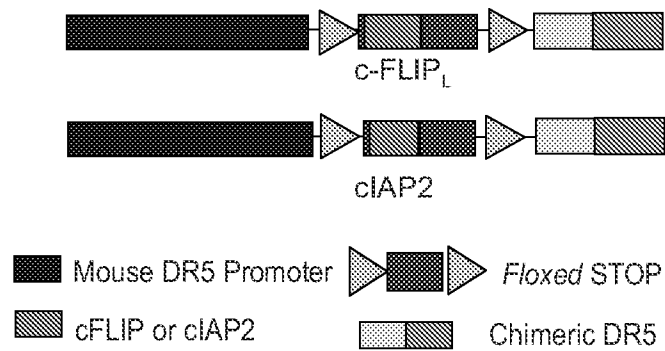


FIG. 18

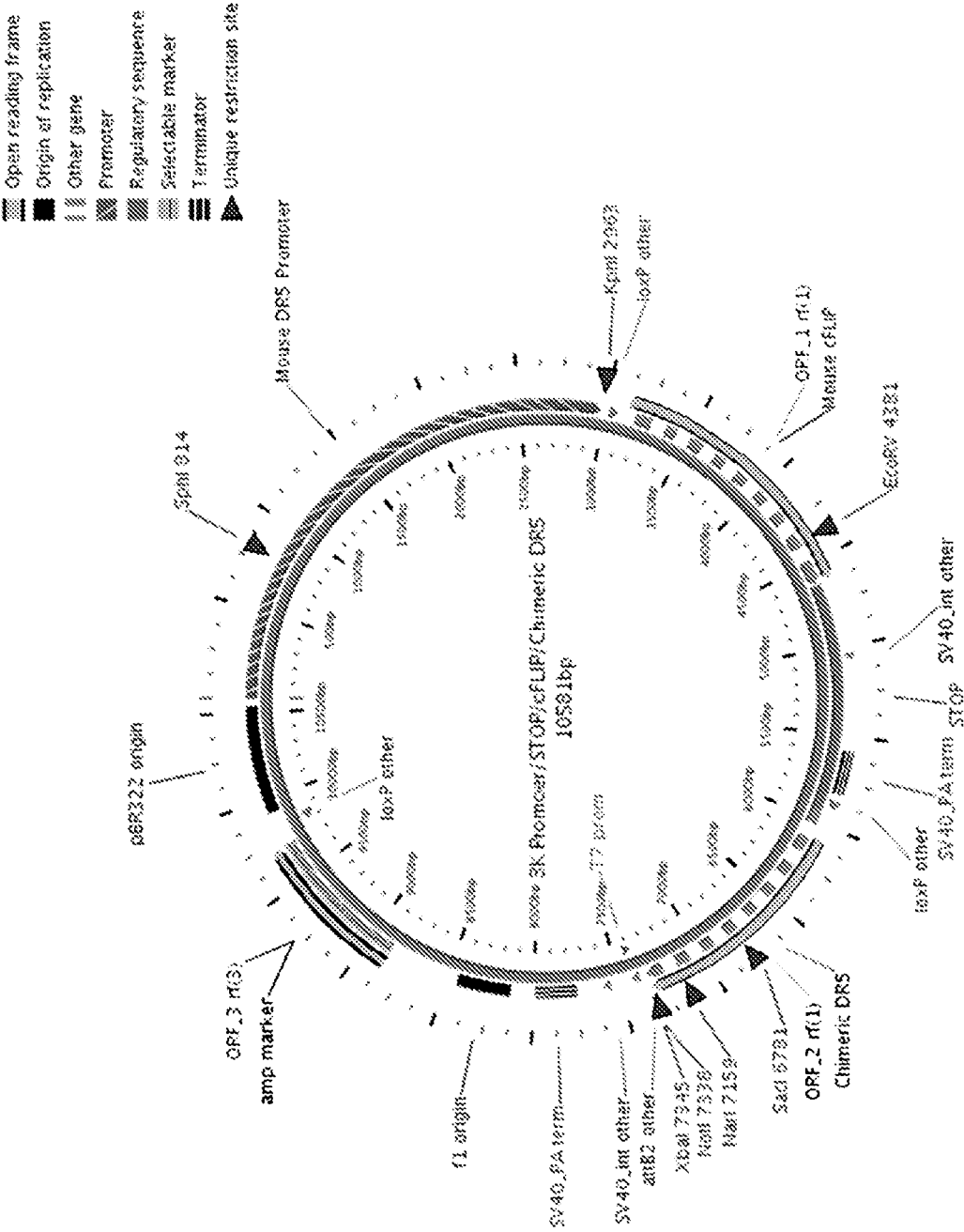


FIG. 19

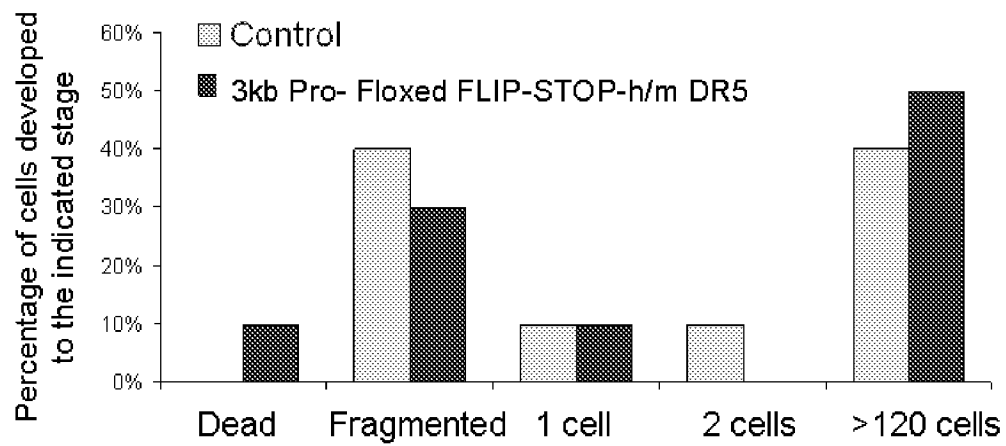
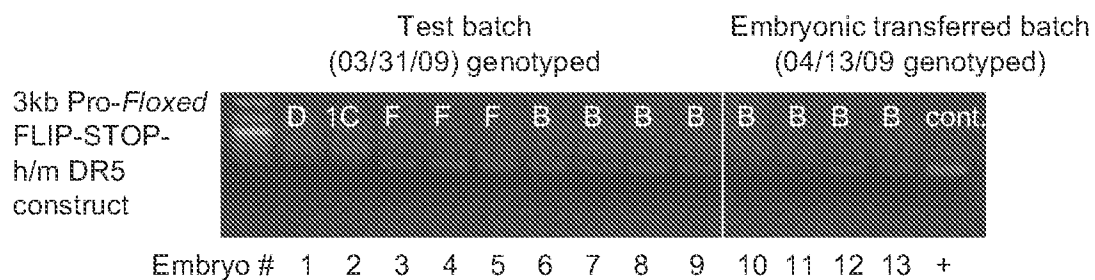


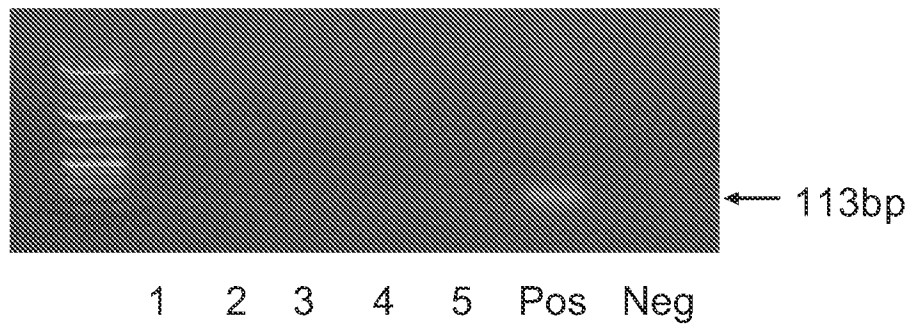
FIG. 20



+ : positive control. Transgene DNA was used as template. -: Negative control

D = dead; 1c = 1 cells; 2c = 2 cells; F = fragmented; B = blastocysts

FIG. 21



DOB: 4/28/2009 Background: B6

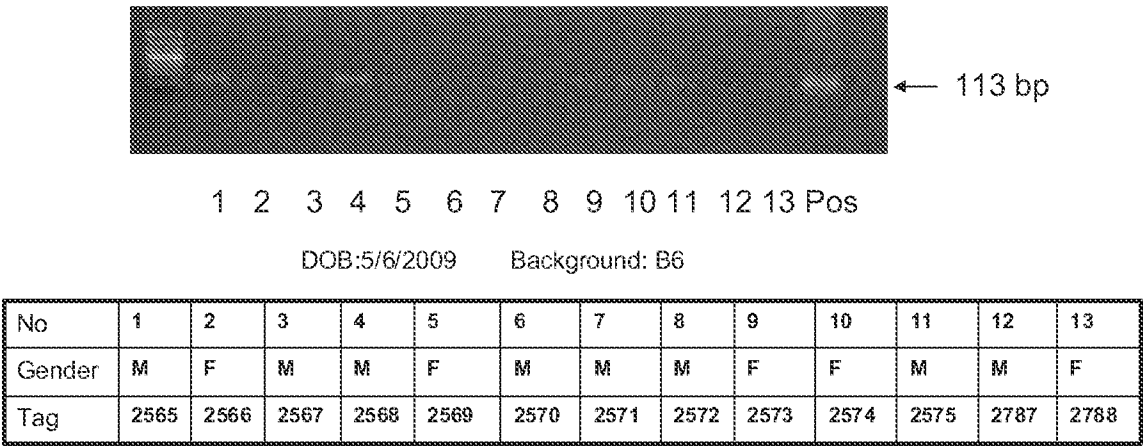
No	1	2	3	4	5
Gender	M	M	M	F	F
Tag	2594	2595	2596	2597	2598

Pos: Positive Control, one of the 3 previous death DR5 + pups

Neg: Negative Control, B6

Primers were designed to target the human DR5 region.

FIG. 22



Pos: positive control, one of the 3 death pups
Primers were designed to target the human DR5 region.

FIG. 23

```

HUMAN      MEQPGQNAFAALSGAREREREGPGFRRARGARFGLEUPRTLVLVVAHVLLIVSAEALITQGD 60
MOUSE      MEFPGEETPTASAAARADHYTF----GLRF---LRFRLILVGFALLAVIQAVFVPTAN 53
          **  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

HUMAN      LAPQGEARAGQQWEESEFEEELCEFPHHIESKDPKICECKNGQWLSYHNS-GLLPGLACDPC 119
MOUSE      EAHNEEGGLQRPEHSEFSGNPILAGQYLSEG--NCKPFRGIGDTYSHSHGLSLGILCTVC 111
          *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

HUMAN      DGEVELSPOTTPTNTVQDCEEGTFHREDSFEMCPKCRIGCCRGHWVVDQTFWSDIDCV 179
MOUSE      EHLFVVEIRONTITNTVQDCEEGTFHREDAPELQSCNN-CTDCEHETISQVWENHICV 170
          *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

HUMAN      HKEGCTHNSGLAFVVEETVTSSPOTFASLGLSCIIIGVTVAHVVLIVAVFVQESLWEX 239
MOUSE      SM-----TANAGSHNLGLIQLAVDPVLLIGALLVETQAGSD 249
          *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

HUMAN      VLPYLKGLCSGGCTDDEPDDESSQPFSAELSVLNEIVSILQPTGVPEQHEVQESAEPTC 299
MOUSE      WLKCIHAGUNE---DSE-----EANGVHSLLEHGTES 293
          *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

HUMAN      VRLSPQESSEHLLERELAESQRKFLVDPANEGQPTETLRQQGFEFASLQVFDGWRFLKE 359
MOUSE      -----TYRLSHWETPEIDSTQKTC-NKLIVPVWENDSADENKTFEYCESLQVFDGWRFLKE 350
          *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

HUMAN      KLGKNDNSINVAAPAAQHEETLVTLINWYKIGKADAVYETLDADETLAGETLAFQKIE 419
MOUSE      KLGKTDNQIQGVFAETLVETRALVQHLKMWKQKQKQKQKQKQKQKQKQKQKQKQKQKQKQK 410
          *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

HUMAN      DALLSGGEFMYLEGNADEGMS----- 440
MOUSE      DYAVEDGRFYNQFSAAGETGTGFEESQCV 391
          *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

```

FIG. 24A

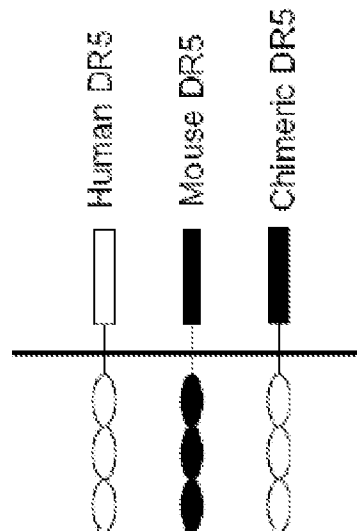


FIG. 24B

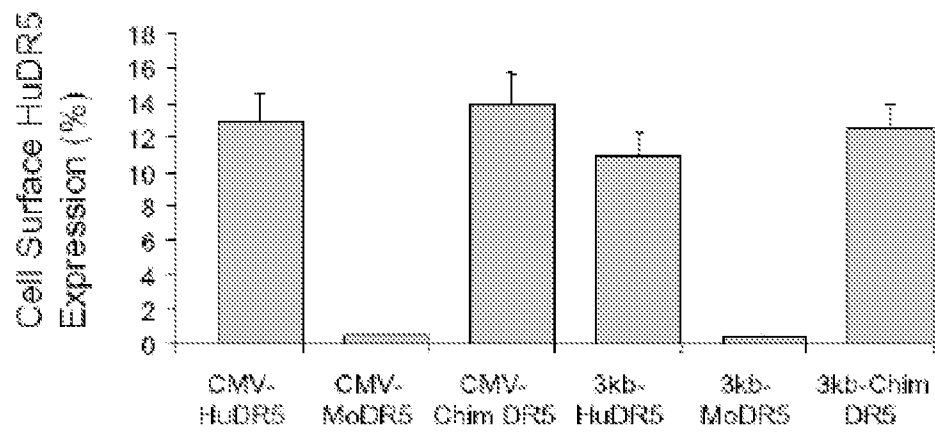
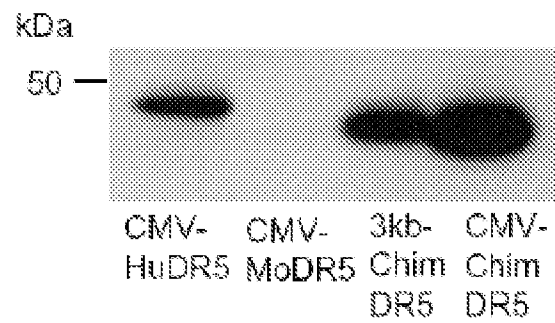


FIG. 24C



IB: anti-human DR5

FIG. 24D

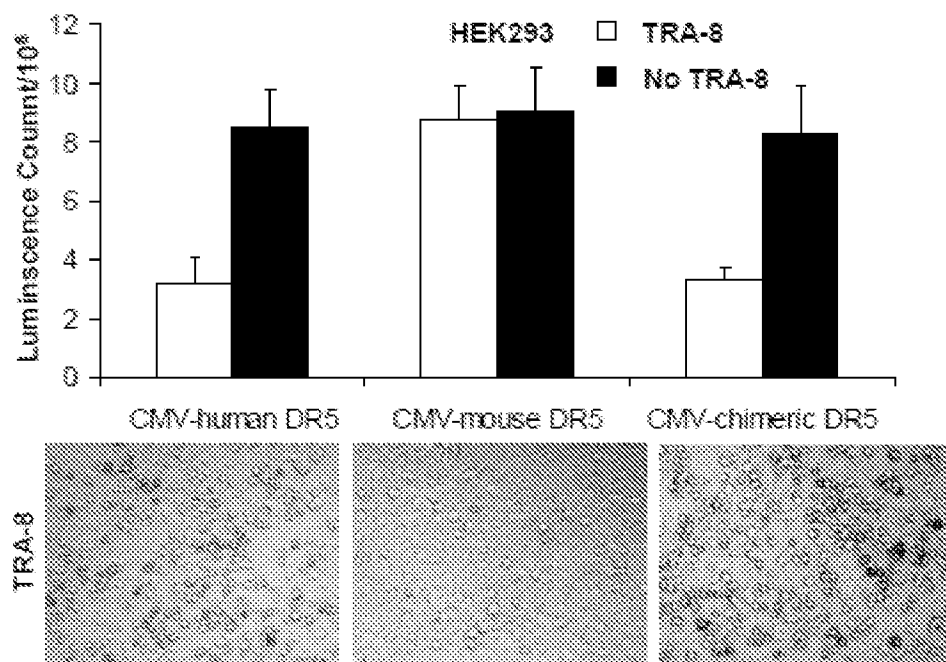


FIG. 25A

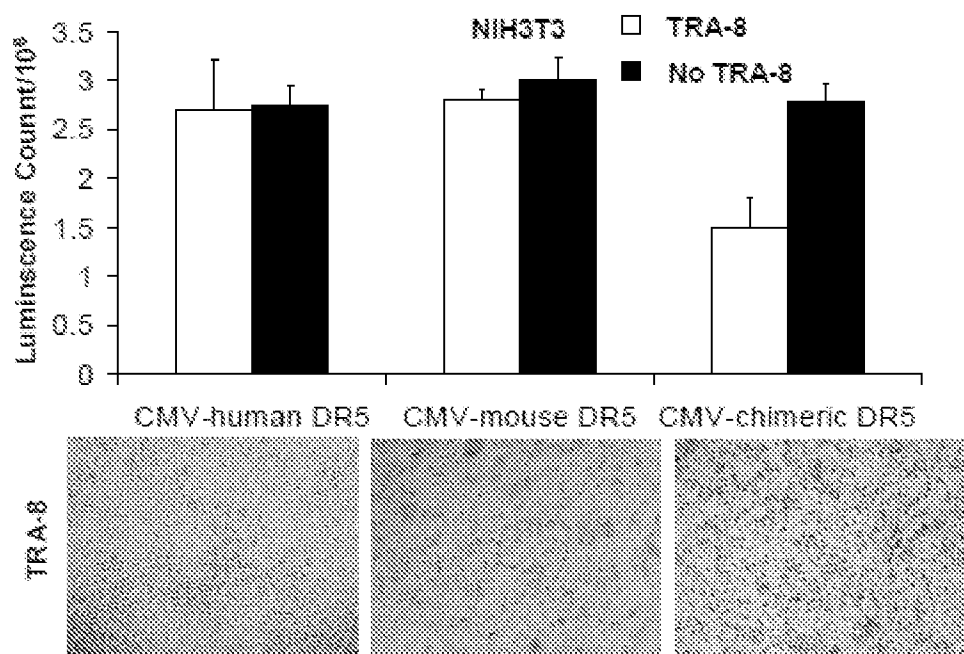


FIG. 25B

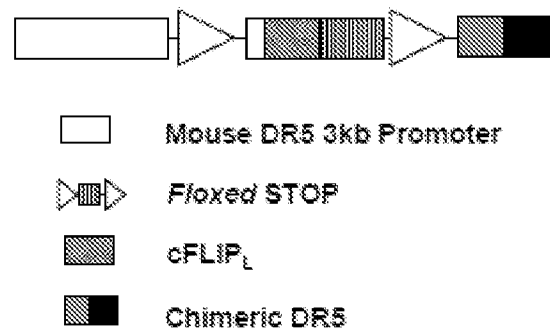


FIG. 25C

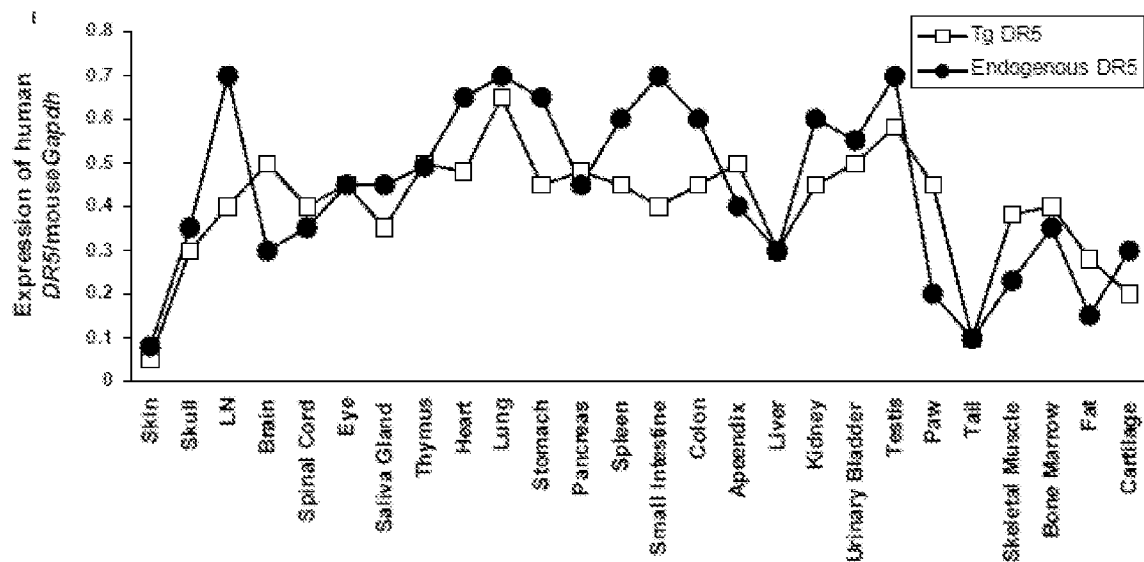


FIG. 25D

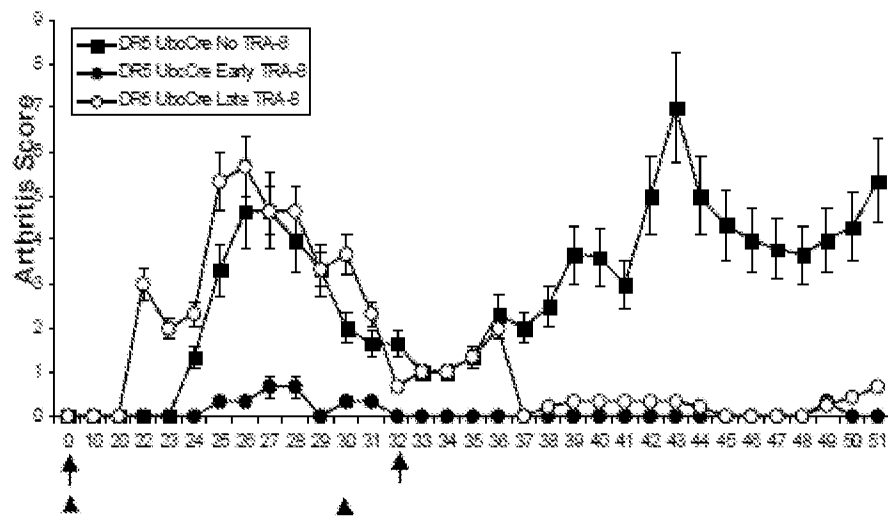


FIG. 26A

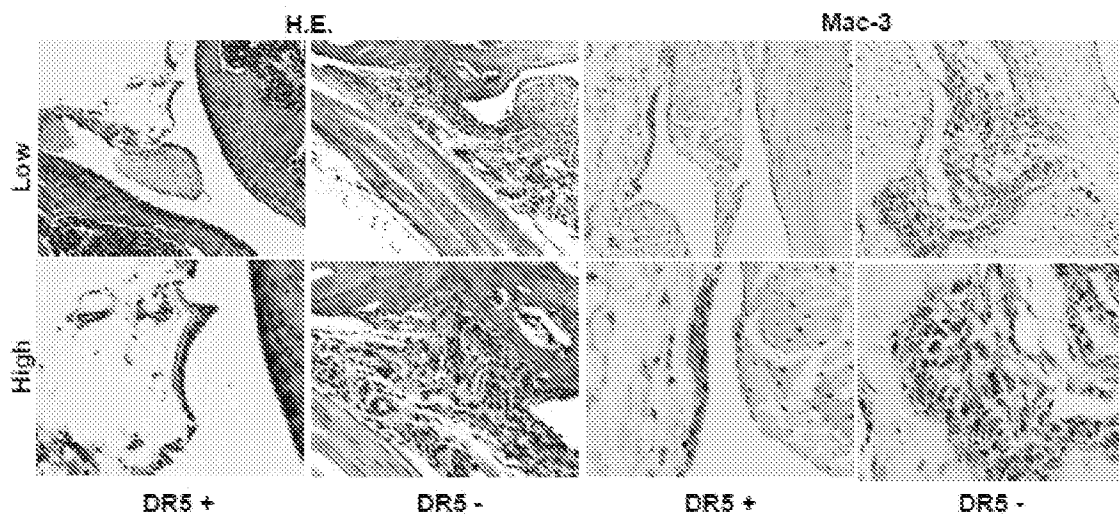


FIG. 26B

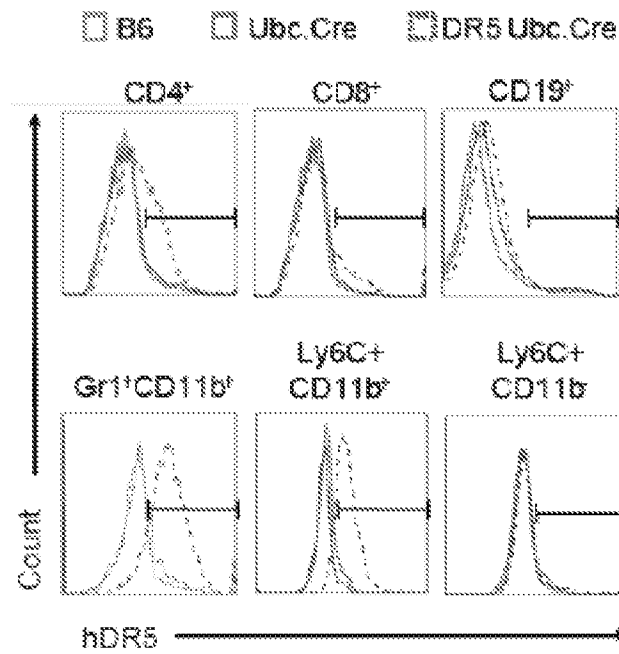


FIG. 26C

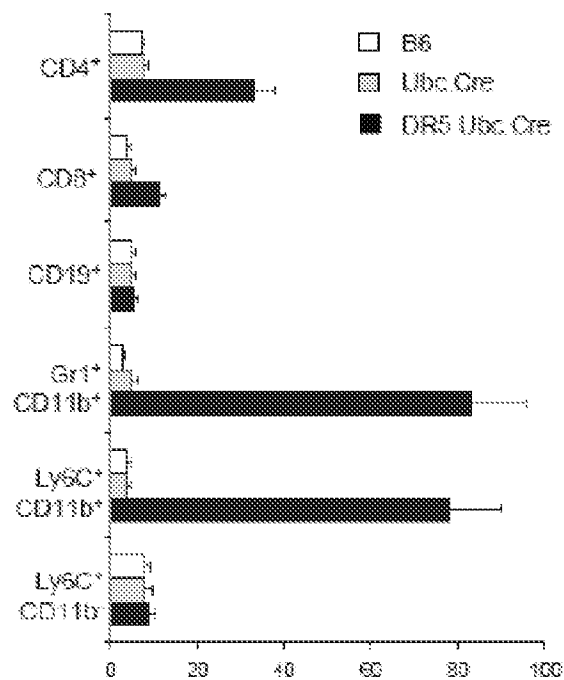


FIG. 26D

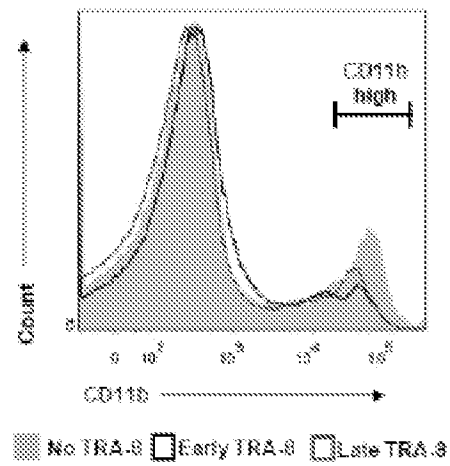


FIG. 26E

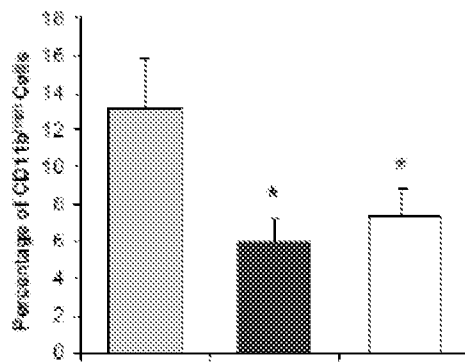


FIG. 26F

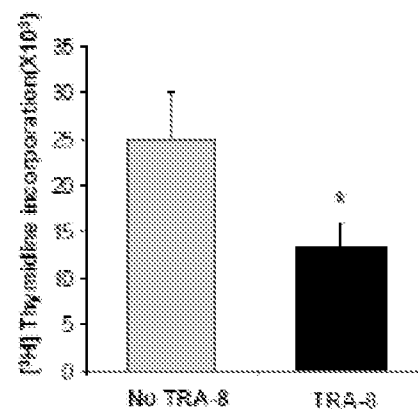


FIG. 26G

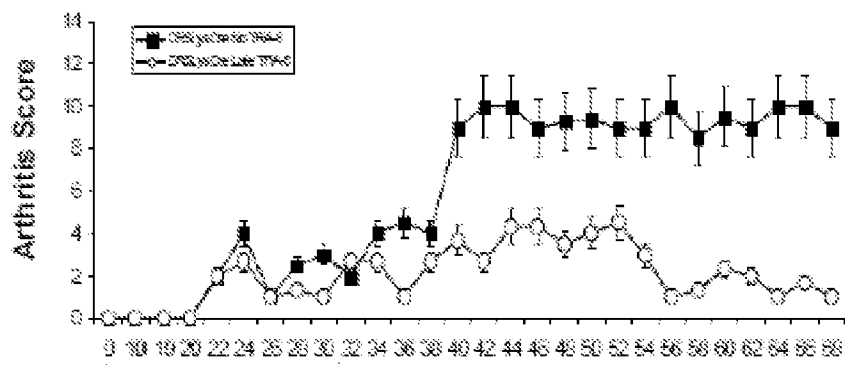


FIG. 27A

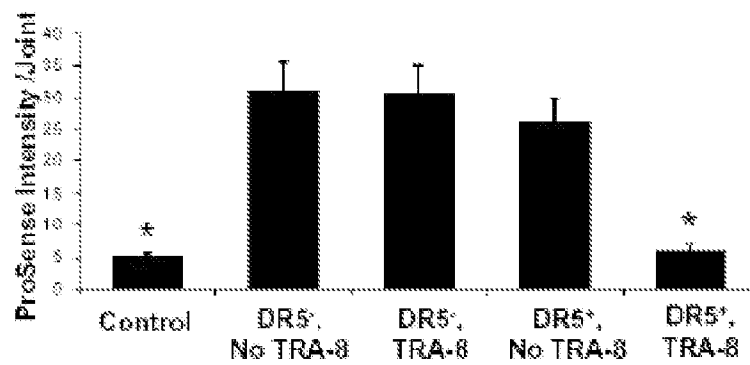
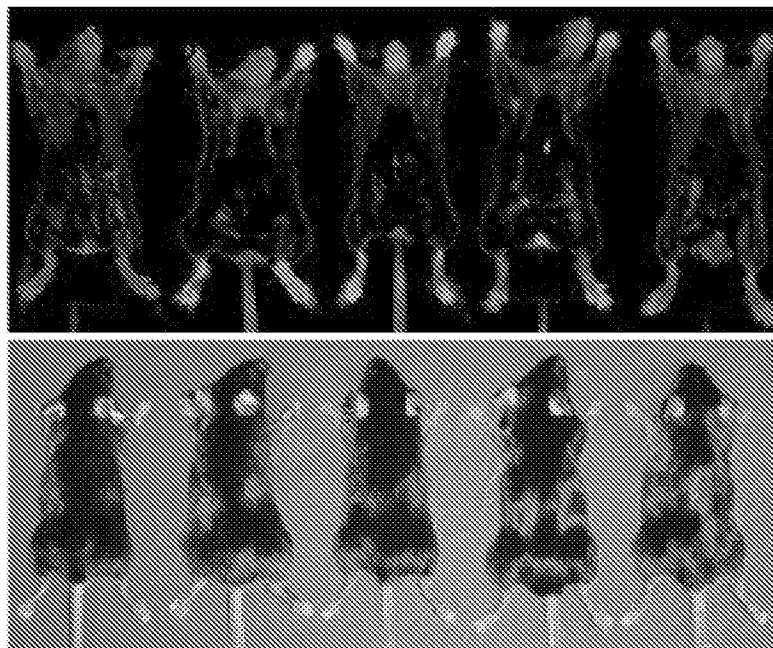


FIG. 27B

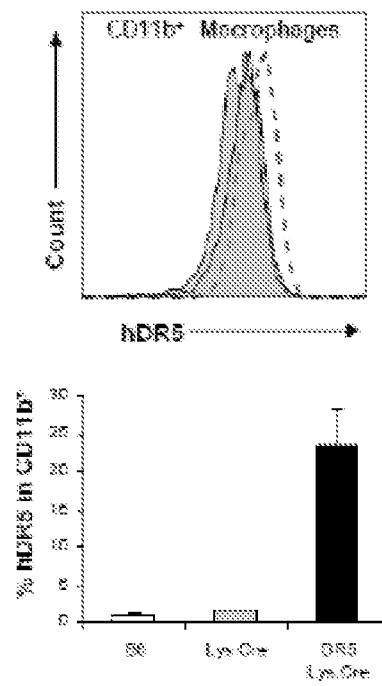


FIG. 27C

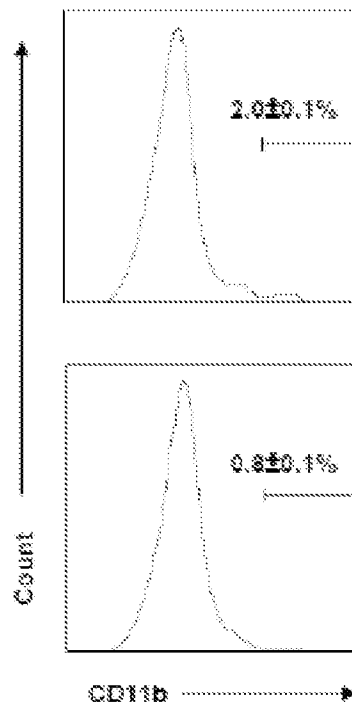


FIG. 27D

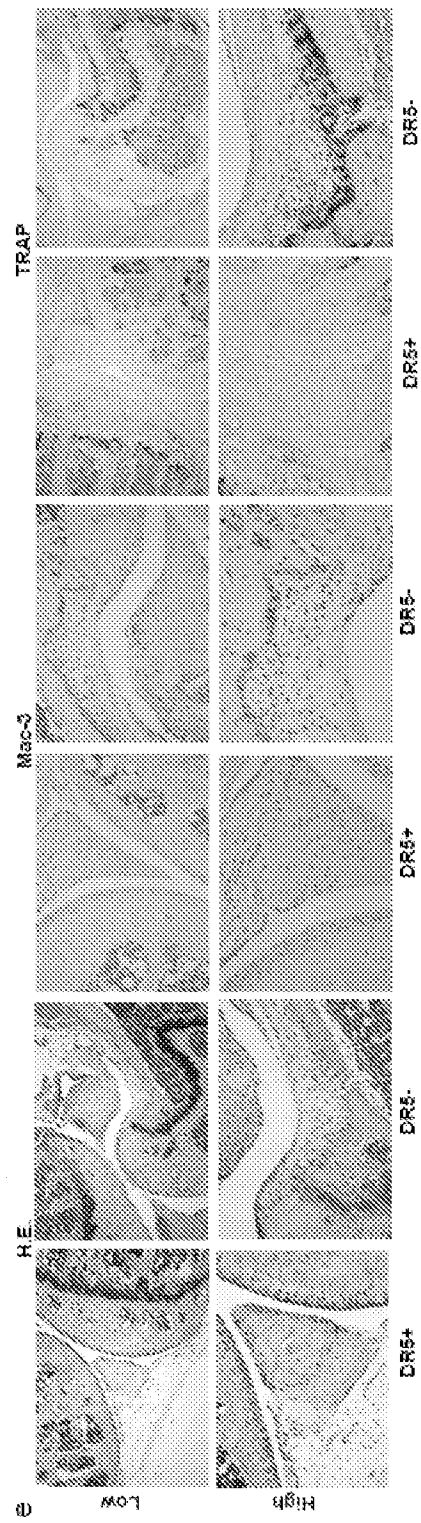


FIG. 27E

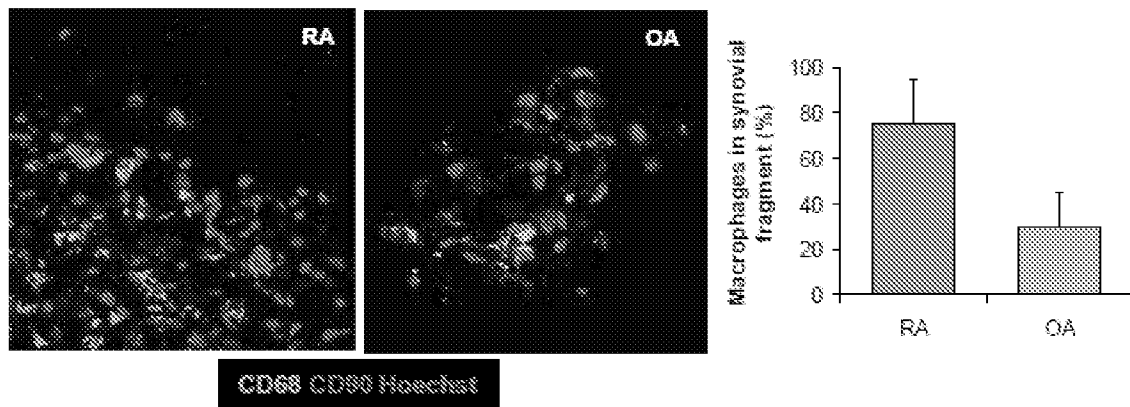


FIG. 28A

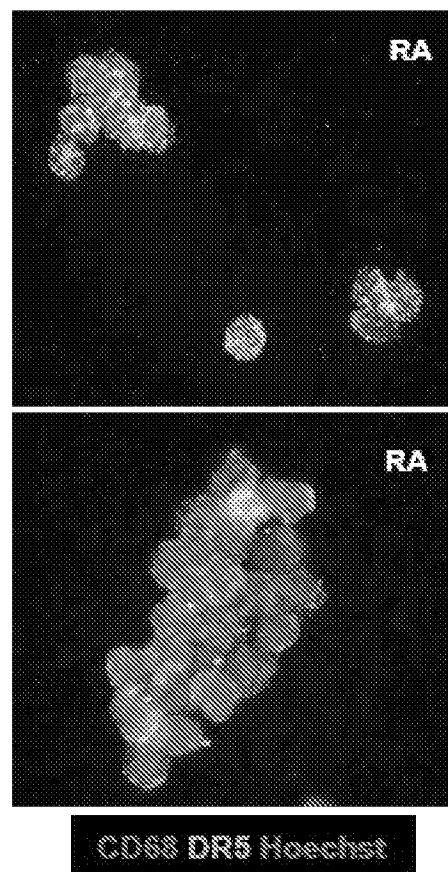


FIG. 28B

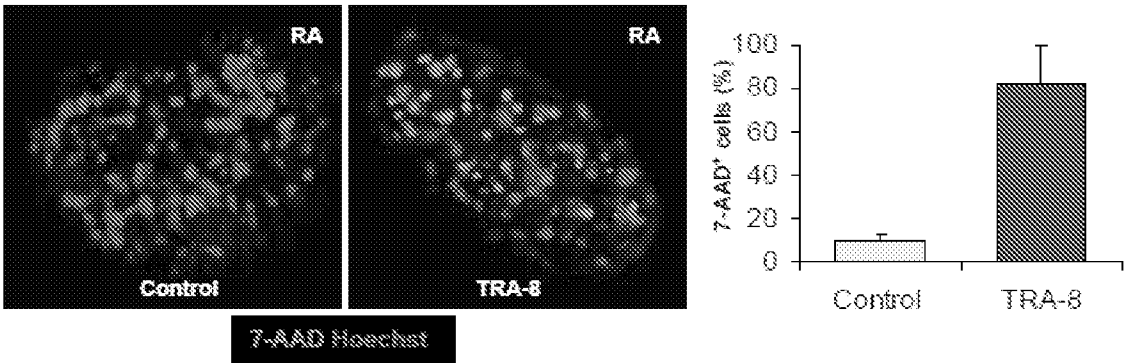


FIG. 28C

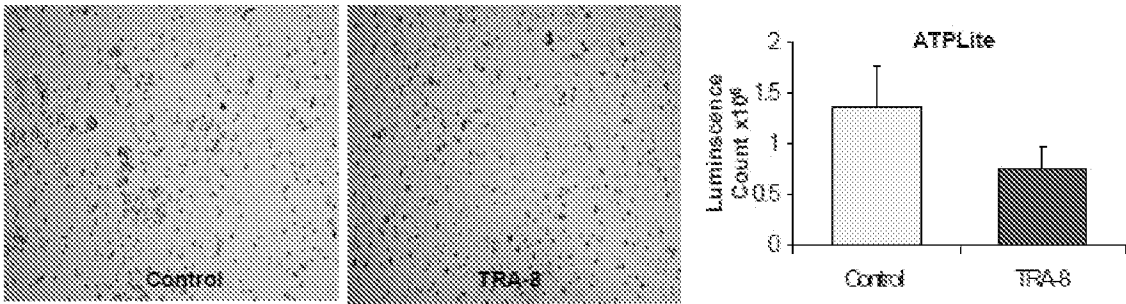


FIG. 28D

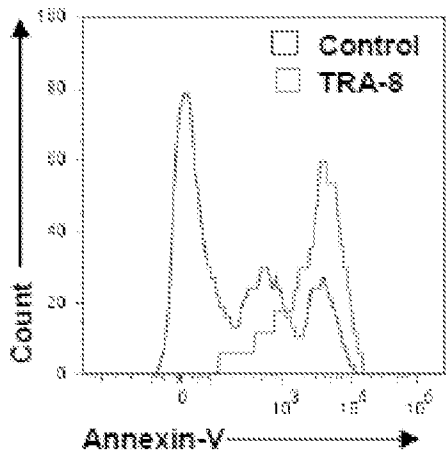


FIG. 28E

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2010/048237

A. CLASSIFICATION OF SUBJECT MATTER INV. A01K67/027 C07K14/705 C07K14/47 C12N15/85 ADD.				
According to International Patent Classification (IPC) or to both national classification and IPC				
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) A01K C07K C12N				
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched				
Electronic data base consulted during the international search (name of data base and, where practical, search terms used) EPO-Internal, BIOSIS, EMBASE, WPI Data				
C. DOCUMENTS CONSIDERED TO BE RELEVANT				
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.		
X	Jun Li, Ping-Ar Yang, Hui-Chen Hsu and John D. Mountz: "A novel human-mouse chimeric death receptor (DR5) molecule mediates TRA-8 induced apoptosis in mouse cell lines" The Journal of Immunology vol. 182, 41.9, 1 April 2009 (2009-04-01), XP002608126 Retrieved from the Internet: URL: http://www.jimmunol.org/cgi/content/meeting_abstract/182/1_MeetingAbstracts/41.9 [retrieved on 2010-11-02] the whole document ----- -/--	1-58		
☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.				
* Special categories of cited documents :				
<table style="width: 100%; border: none;"> <tr> <td style="width: 50%; vertical-align: top; border: none;"> "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed </td> <td style="width: 50%; vertical-align: top; border: none;"> "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family </td> </tr> </table>			"A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family
"A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family			
Date of the actual completion of the international search 4 November 2010	Date of mailing of the international search report 23/11/2010			
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Brero, Alessandro			

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2010/048237

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:
 - a. (means)
☐ on paper
☒ in electronic form
 - b. (time)
☒ in the international application as filed
☐ together with the international application in electronic form
☐ subsequently to this Authority for the purpose of search
2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2010/048237

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	Jun Li, Ping-Ar Yang, Qi Wu Hui-Chen Hsu and John Mountz: "Targeted depletion of macrophages using an anti-human DR5 antibody prevented arthritis in a novel human/mouse chimeric DR5 Tg mouse" The Journal of Immunology vol. 184, 135.29, 1 April 2010 (2010-04-01), XP002608127 Retrieved from the Internet: URL: http://www.jimmunol.org/cgi/content/meeting_abstract/184/1_MeetingAbstracts/135.29 [retrieved on 2010-11-02] the whole document	1-58
A	JAISSER F: "Inducible gene expression and gene modification in transgenic mice." JOURNAL OF THE AMERICAN SOCIETY OF NEPHROLOGY : JASN NOV 2000 LNKD-PUBMED:11065338, vol. 11 Suppl 16, November 2000 (2000-11), pages S95-S100, XP002608128 ISSN: 1046-6673 page s97 - page s99 figure 2B	31
A	ICHIKAWA K ET AL: "TRAIL-R2 (DR5) mediates apoptosis of synovial fibroblasts in rheumatoid arthritis" JOURNAL OF IMMUNOLOGY, AMERICAN ASSOCIATION OF IMMUNOLOGISTS, US, vol. 171, no. 2, 15 July 2003 (2003-07-15), pages 1061-1069, XP002566254 ISSN: 0022-1767 cited in the application the whole document	1-58
A	MARSTERS S A ET AL: "A novel receptor for Apo2L/TRAIL contains a truncated death domain" CURRENT BIOLOGY, CURRENT SCIENCE, GB LNKD-DOI:10.1016/S0960-9822(06)00422-2, vol. 7, no. 12, 1 December 1997 (1997-12-01), pages 1003-1006, XP002087443 ISSN: 0960-9822 * abstract figure 1	1-58
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INTERNATIONAL SEARCH REPORT

International application No

PCT/US2010/048237

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WU GEN SHENG ET AL: "Molecular cloning and functional analysis of the mouse homologue of the KILLER/DR5 tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) death receptor" CANCER RESEARCH, vol. 59, no. 12, 15 June 1999 (1999-06-15) , pages 2770-2775, XP002608129 ISSN: 0008-5472 * abstract figures 1,2 -----	1-58