



(43) International Publication Date
17 September 2015 (17.09.2015)

WIPO | PCT

(10) International Publication Number
WO 2015/136304 A1

(51) International Patent Classification:

A61K 31/132 (2006.01) *A61P 43/00* (2006.01)
A23L 1/00 (2006.01) *A61K 39/00* (2006.01)

(21) International Application Number:

PCT/GB2015/050743

(22) International Filing Date:

13 March 2015 (13.03.2015)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

1404438.2 13 March 2014 (13.03.2014) GB
1420001.8 10 November 2014 (10.11.2014) GB

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(81) Designated States (*unless otherwise indicated, for every
kind of national protection available*): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY,
BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM,
DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,
HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR,
KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG,
MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM,
PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC,
SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN,
TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (*unless otherwise indicated, for every
kind of regional protection available*): ARIPO (BW, GH,
GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ,
TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU,
TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE,
DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU,
LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK,
SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ,
GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— with international search report (Art. 21(3))

(54) Title: POLYAMINE COMPOUNDS AND USES THEREOF

(57) Abstract: The present invention relates to polyamine compounds and their use in enhancing an immune response to an immunogen. The invention also relates to the use of polyamine compounds to treat immunosenescence. The invention further relates to nutraceutical compounds comprising polyamine compounds.



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Polyamine compounds and uses thereof

5 The present invention relates to polyamine compounds and their uses. In particular, the present invention relates to the effect of polyamines on the immune response of a subject to an immunogen.

BACKGROUND

10 An effective and responsive immune system is essential to the maintenance of health, such that the numerous potential pathogens, toxins, aberrant neoplastic cells etc. encountered by a body do not result in pathology or disease. Similarly, such an effective and responsive immune system is required for therapeutic interventions such as vaccines to confer any benefit.

15 If the immune system is suppressed, degraded or degenerate in any way, this can leave an individual susceptible to infection or disease. Such a poorly functioning or ineffective immune system may be caused any of a number of factors, including infection (e.g. HIV), genetic defects (e.g. ADA-SCID),
20 environmental factors (e.g. malnutrition), and aging (immunosenescence).

It is therefore desirable to identify means for restoring or at least partially restoring immune system function in an individual with a poorly functioning immune system. To do so would reduce the risk of those individuals contracting
25 infections or diseases.

One particular, rarely addressed, cause of a poorly functioning immune system is immunosenescence. As individuals age, the cells of the immune system become less responsive to immunogens, with both primary and secondary immune
30 responses demonstrating diminished or aberrant immune responses. In particular the adaptive immune response is decreased with reduced T cell memory formation or/and lower antibody titres.

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This leads to a two-fold problem: as a result of immunosenescence, aged individuals are more susceptible to infection, and at the same time are less responsive to prophylactic or therapeutic vaccination.

Moreover, vaccination even of a healthy individual requires an effective and responsive immune system. It is often desirable to enhance the immune response to a vaccine with a view to improving the protection conferred. This may be achieved by, for example, using multiple doses or boosts of a vaccine, or by using an adjuvant. Examples of commonly-used adjuvants are well-known in the art and include CpG DNA, alum (aluminium phosphate or aluminium hydroxide), and bacterial lipopolysaccharide (LPS). Adjuvants, such as these, work largely through stimulating other aspects of the immune system, often the innate immune system, in order to amplify the adaptive immune response (e.g. CpG or LPS binding to Toll-like receptors (TLRs)). However, adjuvants can lead to reactogenic side-effects such as pain, redness, swelling and scarring as result of activating such potent aspects of the immune system. It is therefore desirable to enhance the immune response independently of TLR triggering with a view to avoiding or reducing such side effects.

The use of polyamines such as spermidine has been shown to prolong the life-span of yeast, flies, nematodes and mice, as well as the life-span of human peripheral blood mononuclear cells (PBMCs) by inhibition of necrotic cell death (Eisenberg *et al.*, Nature Cell. Biol., 11; 1305-1314 (2009)).

SUMMARY OF INVENTION

There is a need to restore or partially restore immune function in individuals exhibiting immunosenescence. There is a particular need to improve the ability of individuals exhibiting immunosenescence to effectively respond to vaccination. It is also desirable to identify a means for improving an individual's response to vaccination but which causes reduced or minimal side-effects.

It has been identified by the present inventors that the use of polyamines, which occur naturally in healthy cells and are required for cell growth, can enhance the

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immune response of an individual to an immunogen such as a vaccine. In particular, the use of polyamines is able to restore the immune response to a vaccine of aged individuals to a level at least equivalent to healthy young controls. Use of polyamines for this purpose has the added advantage of enhancing the immune response with minimal adverse side-effects.

Accordingly, in a first aspect, the invention provides a polyamine for use in the treatment of immunosenescence in a subject. In one embodiment, immunosenescence in a subject is characterised by a refractory immune response to an immunogen, for example a refractory adaptive immune response to an immunogen, such as a refractory T cell response and/or a refractory B cell response.

In one embodiment, the polyamine for use in the treatment of immunosenescence in a subject is administered to the subject prior to the administration to the subject of an immunogen. In another embodiment, the polyamine may be administered following the administration to the subject of an immunogen. In another embodiment, the polyamine may be administered concurrently with the administration to the subject of an immunogen. The polyamine may be administered according to any of these embodiments, alone or in any combination. That is, a polyamine for use in the treatment of immunosenescence in a subject may be administered prior to, and/or concurrently with, and/or following the administration to the subject of an immunogen.

In certain embodiments, the immunogen to which the subject exhibits a refractory immune response and the immunogen administered to the subject is the same immunogen.

In a second aspect, the invention provides a polyamine for use in enhancing the immune response of a subject to an immunogen. In one embodiment, the immune response is an adaptive immune response. That is, the immune response is a T cell (cellular) immune response and/or a B cell (humoral) immune

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response. In a further embodiment, the immune response is a T cell immune response, for example a memory T cell response. In such a further embodiment, the immune response is a CD4 T cell immune response, for example a Th1 and/or a Th2 response. Alternatively the immune response is a CD8 T cell immune response, for example a CD8 memory T cell response.

In certain embodiments, the immune response is a B cell response.

In one embodiment, the polyamine for use in enhancing the immune response of a subject is administered to the subject prior to the administration to the subject of an immunogen. In another embodiment, the polyamine may be administered to the subject following the administration to the subject of an immunogen. In another embodiment, the polyamine may be administered to the subject concurrently with the administration to the subject of an immunogen. The polyamine may be administered according to any of these embodiments, alone or in any combination. That is, a polyamine for use in enhancing the immune response of a subject may be administered prior to, and/or concurrently with, and/or following the administration to the subject of an immunogen.

The following are embodiments of the first or second aspect of the invention. In certain embodiments, the immunogen is a vaccine, preferably a T cell vaccine. In certain embodiments, the polyamine is a naturally-occurring polyamine. In certain embodiments, the polyamine is putrescine, cadaverine, spermidine, or spermine (or an analogue thereof). In a preferred embodiment, the polyamine is spermidine.

In certain embodiments, the polyamine is administered to the subject in solution, preferably aqueous solution. In certain embodiments the polyamine is administered to the subject orally, intravenously, intramuscularly, intraperitoneally, intradermally, subcutaneously, by gavage, bronchoscope, nebuliser, or suppository.

In certain embodiments, the polyamine is administered to the subject at a concentration of 1-10 mM, preferably 1-5 mM, preferably 5 mM. In certain embodiments, the polyamine is administered to the subject at least once a day for 1 to 200 days, optionally 1 to 110 days, optionally 1 to 90 days, optionally 1 to 52 days, optionally 1 to 21 days, optionally 1 day (that is, wherein the polyamine is administered once). In an alternative embodiment the polyamine is administered to the subject at least once a week for 1 to 30 weeks, optionally 1 to 16 weeks, optionally 1 to 13 weeks, optionally 1 to 8 weeks, optionally 1 to 3 weeks, optionally 1 week.

In a third aspect, the invention provides a method of enhancing the immune response of a subject to an immunogen, the method comprising (i) administering a polyamine to the subject; and (ii) administering the immunogen to the subject. In certain embodiments step (i) is performed prior to and/or concurrently with and/or following step (ii).

In certain embodiments, the method enhances the adaptive immune response, preferably the T cell immune response. In preferred such embodiments, the method enhances the memory T cell immune response. In certain embodiments, the method enhances the CD8 T cell response, for example the memory CD8 T cell response, and/or the CD4 T cell response, for example the CD4 Th1 response and/or the CD4 Th2 response.

In certain embodiments, the immunogen is a vaccine, preferably a T cell vaccine. In certain embodiments, the polyamine is a naturally-occurring polyamine. In certain embodiments, the polyamine is putrescine, cadaverine, spermidine, or spermine. In a preferred embodiment, the polyamine is spermidine.

In certain embodiments, the polyamine is administered to the subject in solution, preferably aqueous solution. In certain embodiments the polyamine is administered to the subject orally, intravenously, intramuscularly, intraperitoneally, intradermally, subcutaneously, by gavage, bronchoscope, nebuliser, or suppository.

In certain embodiments, the polyamine is administered to the subject at a concentration of 1-10 mM, preferably 1-5 mM, preferably 5 mM. In certain embodiments, the polyamine is administered to the subject at least once a day for 1 to 200 days, optionally 1 to 110 days, optionally 1 to 90 days, optionally 1 to 52 days, optionally 1 to 21 days, optionally 1 day (that is, wherein the polyamine is administered once). In an alternative embodiment the polyamine is administered to the subject at least once a week for 1 to 30 weeks, optionally 1 to 16 weeks, optionally 1 to 13 weeks, optionally 1 to 8 weeks, optionally 1 to 3 weeks, optionally 1 week

In a fourth aspect, the invention provides a nutraceutical preparation comprising a polyamine, wherein said preparation is suitable for the treatment of immunosenescence. In certain embodiments, the nutraceutical preparation is suitable for oral administration, preferably ingestion. Accordingly, the invention provides an ingestible nutraceutical preparation comprising a polyamine, wherein said preparation is suitable for the treatment of immunosenescence. In certain preferred embodiments the nutraceutical preparation is a beverage. In certain embodiments the nutraceutical preparation is a dairy-based preparation, for example a yoghurt. In certain embodiments, the preparation comprises the polyamine in a concentration of 1-10 mM, preferably 1-5 mM, preferably 5 mM.

In certain embodiments the invention provides a nutraceutical preparation according to the fourth aspect for use in the treatment of immunosenescence. In certain such embodiments, the preparation is administered at least once a day for 1 to 200 days, optionally 1 to 110 days, optionally 1 to 90 days, optionally 1 to 52 days, optionally 1 to 21 days, optionally 1 day, optionally wherein the preparation is administered once. In certain alternative embodiments, the preparation is administered weekly for 1 to 30 weeks, optionally 1 to 16 weeks, optionally 1 to 13 weeks, optionally 1 to 8 weeks, optionally 1 to 3 weeks, optionally 1 week.

FIGURES

Figure 1. *Polyamine treatment enhances the CD8⁺ T cell response to vaccination in aged mice.* CD8⁺ T cell kinetics in 14 week old and 25 month old wildtype and CD4-Atg7^{-/-} mice were tracked in the blood by tetramer following influenza vaccination. Mice were vaccinated on day 0 and day 22. Spermidine was administered in the drinking water 21 days prior to the first vaccination through to the experimental endpoint.

Figure 2. *Polyamine treatment improves the CD8⁺ T cell response in the blood to influenza challenge in aged mice.* Eight week old, 25 month old wildtype, and 25 month old CD4-Atg7^{-/-} mice were vaccinated on day 0 and day 22. Thirty days later they were challenged with 32 HAU wildtype X31 Influenza virus intranasally. The frequency of CD8⁺ T cells specific for NP₃₆₆₋₃₇₄ was then assessed in the blood by tetramer on day 9 post-challenge. Spermidine was administered in the drinking water 21 days prior to the first vaccination through to the experimental endpoint.

Figure 3. *Polyamine treatment improves the CD8⁺ T cell response in the lungs to influenza challenge in aged mice.* Eight week old, 25 month old wildtype, and 25 month old CD4-Atg7^{-/-} mice were vaccinated on day 0 and day 22. Thirty days later they were challenged with 32 HAU wildtype X31 Influenza virus intranasally. The frequency (**A**) and absolute counts (**B**) of CD8⁺ T cells specific for NP₃₆₆₋₃₇₄ was assessed in the lungs by tetramer on day 9 post-influenza challenge. Spermidine was administered in the drinking water 21 days prior to the first vaccination through to the experimental endpoint.

Figure 4. *Polyamine treatment improves the CD8⁺ T cell response to influenza infection in young mice.* Eight week old mice were treated with spermidine in the drinking water at a concentration of 5mM for 8 weeks then infected with 0.001 X31 (H3N2) influenza. Data shown is the frequency CD8⁺ T cells specific for NP₃₆₆₋₃₇₄ in the lungs by tetramer on day 7 post infection.

Figure 5. 8 week old mice were immunised intranasally with the influenza vaccine S-Flu. On day 7 the vaccine specific CD8⁺ T cell response was assessed in the lungs. From day 0 to day 7 spermidine was administered in the drinking water at a concentration of 5mM. Shown is the frequency of CD8⁺ T cells that are specific for the Influenza epitope NP₃₆₆₋₃₇₄.

Figure 6. 8 week old mice were immunised intranasally with the influenza vaccine S-Flu. On day 14 the vaccine specific CD8⁺ T cell response was assessed in the lungs. In one group of mice, 100 µg of spermidine was administered intraperitoneally every other day from day 0 to day 14, with the first administration performed concurrently with vaccination (Figure 6A). For another group of mice, 500 µg of spermidine was administered intraperitoneally concurrently with the vaccination. Shown is the frequency of CD8⁺ T cells that are specific for the Influenza epitope NP₃₆₆₋₃₇₄.

Figure 7. *Lymphopenia induces homeostatic proliferation and an activated CD8⁺ T cell phenotype in T-Atg7^{-/-} mice.*

a) Frequency of mature single positive CD4⁺ and CD8⁺ T cells in thymi of six week old mice (n=4), representative FACS plot of 3 independent experiments. **b)** Flow cytometric analysis of CD4⁺ and CD8⁺ T cell frequencies in blood and lymph nodes of T-Atg7^{-/-} and wild type mice (dot plots depict staining in blood). Quantitative analyses are representative of six independent experiments, **p*<0.05 (n=4). **c)** Absolute counts of CD4⁺ and CD8⁺ T cells in blood of T-Atg7^{-/-} and WT mice over time (n=4). **d)** Percentage of CD44^{hi} cells in the splenic CD8⁺ T cell compartment of T-Atg7^{-/-} and WT mice. Bar graphs depict the frequency of gated cells (representative of seven independent experiments), **p*<0.05 (n=4). **e)** Percentage of splenic CD8⁺ T cells positive for CD62L in T-Atg7^{-/-} and WT mice. Data are representative of three independent experiments, **p*<0.05, (n=4). **f)** Percentage of CD44^{lo} and CD44^{hi} CD8⁺ T cells expressing Ki-67 in the spleen of T-Atg7^{-/-} and WT mice. Bar graphs are representative of three independent experiments, **P*<0.05 (n=4). **g)** Frequency of splenic CD8⁺ T cells expressing CD24 in T-Atg7^{-/-} and WT mice. Bar graph is representative of two independent experiments, **P*<0.05 (n=4). **h)** Percentage of donor-derived CD62L⁺CD8⁺ T cells. Lethally irradiated CD45.1 hosts reconstituted with a 1:1 mix of T-Atg7^{-/-} or

WT BM (both CD45.2) with CD45.1 wild type BM. Controls were WT and T-*Atg7*^{-/-} mice; **P*<0.05 (n=4). **i)** Frequency of donor-derived CD45.2⁺ CD8⁺ T cells in the spleen that are CD44^{hi}. Controls were normal WT and T-*Atg7*^{-/-} mice, **P*<0.05 (n=4). All values are mean ± s.e.m and all statistical analyses are Mann-Whitney U-tests.

Figure 8.

a) *Atg7* gene expression in *Atg7*^{+/+} and *Atg7*^{-/-} T cells. T cells were isolated from the spleens of WT and T-*Atg7*^{-/-} mice by flow cytometry. mRNA was extracted and *Atg7* gene expression measured by q-PCR. Bar graph shows relative *Atg7* expression in T cells from T-*Atg7*^{-/-} mice compared to T cells from WT mice. *****p*<0.0001 by Student's t-test. **b)** Protein was purified from pooled MACS-sorted CD8⁺ T cells from WT and T-*Atg7*^{-/-} mice and Western Blotted for ATG7. GAPDH was used as a loading control (n=6 pooled mice) **c)** LC3 Spot count in WT and *Atg7*^{-/-} CD8⁺ T cells. Splenocytes from WT and T-*Atg7*^{-/-} mice were untreated or treated for 2 hours with an autophagy flux inhibitor before staining for relevant cell surface markers and LC3-II. LC3 spot count was assessed using ImageStream software. Quantification is gated on CD8⁺ T cells. Right panel depicts example LC3 spot count images (x60 magnification). **p*=0.0132, ****P*=0.0002 as determined by Student t-test (n=4) **d)** Autophagy flux in WT and *Atg7*^{-/-} CD8⁺ T cells. Purified CD8⁺ T cells were untreated or treated with bafilomycin for 6 hours before whole protein extraction and western blot for LC3. **e)** Percentage of CD44^{hi} CD62L⁻ and CD44^{hi} CD62L⁺ cells in the splenic CD8⁺ T cell compartment of T-*Atg7*^{-/-} and WT mice. Representative FACS plots of 3 independent experiments are shown, ****p*=0.0003 as determined by Mann-Whitney U-test (n=9).

Figure 9

a) Flow cytometric analysis of CD4⁺ and CD8⁺ T cell frequencies in the spleen of WT and Vav-*Atg5*^{-/-} mice. Quantitative analyses are representative of two independent experiments, ***p*<0.01 (n=5). **b)** Frequency of CD44^{hi} cells within the CD8⁺ T cell subset of WT and Vav-*Atg5*^{-/-} mice. Data are representative of two independent experiments, ***p*<0.01 (n=5). **c)** BM reconstitution in BM chimera mice. Nine weeks after marrow transplantation the frequency of donor CD45.2

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cells was assessed in the spleen by flow cytometry. Quantified is the frequency of splenocytes expressing CD45.2 in BM chimera mice and in normal WT and T-*Atg7*^{-/-} mice that acted as controls. **d)** Frequency of CD8⁺ T cells that are CD45.2 donor-derived in BM chimera mice. Example dot plots are gated on CD8⁺ T cells. Quantified is the frequency of CD8⁺ T cells that express CD45.2 in BM chimera and in normal WT and T-*Atg7*^{-/-} mice that acted as controls. **p*<0.05 (n=4). Data are mean ± s.e.m and all statistical analyses are Mann-Whitney U-tests.

Figure 10. Normal effector CD8⁺ T cell responses to viral infection but defective memory CD8⁺ T cell formation in T-*Atg7*^{-/-} mice.

a) Effector CD8⁺ T cell response to influenza in WT and T-*Atg7*^{-/-} mice. Mice were immunized intranasally with 0.00032 HAU PR8 influenza. On day 10, antigen-specific CD8⁺ T cells to Nucleoprotein (NP) was assessed with tetramer in lungs. Dot plots show examples of tetramer staining gated on CD8⁺ T cells. Bar graph indicates percentage of CD8⁺ T cells specific for NP (n=5-6) and is representative of three independent experiments. **b)** Effector CD8⁺ T cell response to MCMV in WT and T-*Atg7*^{-/-} mice. CD8⁺ T cells from blood were stained with m45 tetramer on day 7 post-infection. Dot plots show m45-tetramer⁺ cells gated on CD8⁺ T cells. Bar graph indicates % CD8⁺ T cells specific for m45 (n=4-5) and is representative of three independent experiments. **c)** CD8⁺ T_{mem} response to influenza. WT and T-*Atg7*^{-/-} mice immunized as in a) and the antigen-specific CD8⁺ T cell response was assessed in lungs on day 50 by tetramer. **p*<0.05, by Mann-Whitney U-test (n=4). Dot plots are gated on CD8⁺ T cells. Bar graph is representative of three independent experiments. **d)** CD8⁺ T_{mem} response to MCMV. Lung CD8⁺ T cells on day 65 post-infection were stained with IE3-tetramer. Dot plots are gated on CD8⁺ T cells. Quantitation depicts frequency of IE3 specific CD8⁺ T cells. **p*<0.05, by Mann-Whitney U-test (n=4). Data are representative of two independent experiments. **e)** CD8⁺ T cell kinetics to influenza infection. WT and T-*Atg7*^{-/-} mice were immunized as in (a) and CD8⁺ T cell response tracked over time in blood by tetramer. Y-axis shows frequency of NP-specific CD8⁺ T cells. **f)** CD8⁺ T cell kinetics to MCMV infection. CD8⁺ T cell response to epitopes m38 (left panel) and IE3 (right panel) were tracked over time in blood by tetramer in WT and T-*Atg7*^{-/-} mice. Y-axis indicates the

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percentage of CD8⁺ T cells that are m38-specific. **g)** Influenza virus titres. WT and T-*Atg7*^{-/-} mice were culled at days 3 and 6 post-immunization with PR8 and lungs collected and snap frozen in liquid nitrogen. Virus titres were determined using MDCK-SIAT1 cells. ****p*=0.0002 as determined by Student t-test (n=4) **h)** WT mice were immunized with PR8 influenza as in (a). CD8⁺ T cells from spleen were stained with CytoID at day 9 post-infection and assessed by flow cytometry. Histograms show examples of CD44^{lo} CD8⁺ T cells from unimmunized mice (filled grey line) and in NP-specific CD8⁺ T cells from immunized mice (open black line). Quantification is by mean fluorescence intensity (MFI) of CytoID on gated indicated cell population and representative of two independent experiments. *****p*<0.0001 by Student t-test (n=6). **i)** Autophagy levels by CytoID staining on CD8⁺ T cells from the lungs on day 9 post-infection as in (g). Histograms provide examples of CytoID staining, gated on CD8⁺ T cells in lungs of immunized mice (solid grey lines) and gated on NP-tetramer-specific CD8⁺ T cells from immunized mice (open black lines). Quantification is by mean fluorescence intensity (MFI) of CytoID on gated indicated cell population and representative of two independent experiments. *****p*<0.0001 by Student t-test (n=4-8). All values are mean ± s.e.m.

Figure 11

a) Effector CD8⁺ T cell absolute counts in WT and T-*Atg7*^{-/-} mice. Quantification of the absolute number of CD8⁺ NP-tetramer⁺ T cells was determined in the lungs on day 10 of PR8 influenza infection ***p*=0.0084 by Student t-test (n=6-9). **b)** Frequency of m45-specific CD8⁺ T cells in the liver of MCMV-immunized WT and T-*Atg7*^{-/-} mice on day 100 post-infection. Dot plots are gated on CD8⁺ T cells; bar graphs depict the percentage of CD8⁺ T cells that are m45-tetramer⁺. All values are mean ± s.e.m.

Figure 12. Loss of memory CD8⁺ T cell formation in the absence of *Atg7* is not due to lymphopenia, poor CD4⁺ T cell help, exhaustion or defective cytokine receptor expression

a) CD8⁺ T_{mem} response to influenza in mixed BM chimeras, generated as in (1h). Mice were immunized with PR8 influenza and the CD8⁺ T_{mem} response of the CD45.2 donor to NP was assessed in lungs by tetramer on day 40. Quantitation shows frequency of (donor) CD45.2⁺ CD8⁺ T cells that are NP-specific (n=5-6).

** $p < 0.01$, by Mann-Whitney U-test ($n = 4-7$). **b)** SLEC and MPEC populations in the *Atg7^{+/+}* and *Atg7^{-/-}* antigen-specific CD8⁺ T cell pool. Mixed BM chimera generated as in (1h), were immunized with MCMV eight weeks after transplantation. Dot plots show example of KLRG1 and CD127 expression on gated CD45.2⁺ m45-tetramer⁺ CD8⁺ T cells on day 10 post-infection. Upper bar graph depicts the % of CD45.2⁺ m45-tetramer⁺ CD8⁺ T cells that are CD127⁻ KLRG1⁺ (SLECs). Lower bar graph shows the % of CD127⁺ KLRG1⁻ (MPECs) in the same population. * $p < 0.05$, by Mann-Whitney U-test ($n = 4-7$). **c)** Markers of exhaustion on *Atg7^{-/-}* MCMV-specific CD8⁺ T cells on MCMV challenged BM chimera. Dot plots depict example of PD-1 and TIM-3 staining on gated CD45.2⁺ m45-tetramer⁺ CD8⁺ T cells. Bar graph quantifies the percentage of (donor) CD45.2⁺ m45-tetramer⁺ CD8⁺ T cells that are PD-1⁺ TIM-3⁺ at day 10 post-infection. * $p < 0.05$, by Mann-Whitney U-test ($n = 4-7$). **d)** CD127 expression on *Atg7^{-/-}* MCMV-specific CD8⁺ T cells in MCMV challenged BM chimeras. Examples of CD127 staining on gated CD45.2⁺ m45-tetramer⁺ CD8⁺ T cells from spleen on day 10 post-infection are shown * $p < 0.05$, by Mann-Whitney U-test ($n = 4-7$). **e)** IL-15R α expression on splenic *Atg7^{-/-}* MCMV-specific CD8⁺ T cells in MCMV challenged BM chimeras. Histograms depict IL-15R α expression in CD44^{lo} CD8⁺ T cells from unimmunized mice (grey dotted line), *Atg7^{+/+}* CD45.2⁺ m45-tetramer CD8⁺ T cells (grey filled line) and *Atg7^{-/-}* CD45.2⁺ m45-tetramer CD8⁺ T cells (black line). The left histogram shows expression in control normal WT and T-*Atg7^{-/-}* mice, the right histogram indicates staining in donor CD45.2⁺ cells from BM chimera mice. Quantified is IL-15R α mean fluorescence intensity on gated CD45.2⁺ m45-tetramer CD8⁺ T cells ($n = 4-7$). All values are mean $\pm$ s.e.m.

Figure 13.

a) IRF4 expression in *Atg7^{-/-}* and *Atg7^{+/+}* splenic m45-specific CD8⁺ T cells on day 9, 15 and 22 post-infection. As a control, IRF4 was also measured in CD44^{lo} CD8⁺ T cells from unimmunized mice (naïve). Quantification shows IRF4 mean fluorescence intensity from gated m45-tetramer⁺ CD8⁺ T cells and CD44^{lo} CD8⁺ T cells (naïve). Statistics – Student's t-test ($n = 4-5$). **b)** EOMES expression in *Atg7^{-/-}* and *Atg7^{+/+}* antigen-specific CD8⁺ T cells. WT and T-*Atg7^{-/-}* mice were immunized with MCMV and EOMES expression was measured in m45-specific CD8⁺ T cells on day 9, 15, and 22. As a control, EOMES was also measured in CD44^{lo} CD8⁺ T

cells from unimmunized mice (naïve). Quantification shows EOMES mean fluorescence intensity on gated m45-tetramer⁺ CD8⁺ T cells and CD44^{lo} CD8⁺ T cells (naïve) (n=4-5).

Figure 14. *Atg7*^{-/-} memory CD8⁺ T cells show increased mitochondrial content, reactive oxygen species, apoptosis and fail to down-regulate GLUT-1.

a) The spleens of unimmunized and MCMV-infected mice were stained with the apoptotic marker Annexin V and a dead cell dye that stains cells with disrupted membranes on the time points indicated. Apoptotic cells were defined as dead cell dye-negative Annexin V⁺. Dot plots are gated on either CD44^{lo} CD8⁺ T cells (naïve) or m45-tetramer⁺ CD8⁺ T cells. **p*<0.05, by Mann-Whitney U-test (n=4-5). **b)** Mitochondrial volume by MitoTracker Green in Tetramer⁺ CD8⁺ T cells from WT and T-*Atg7*^{-/-} mice. Spleens from MCMV-immunized mice were stained with MitoTracker Green on day 15 post-infection. Quantification depicts mean fluorescence intensity on m45-tetramer⁺ CD8⁺ T cells and is representative of three independent experiments. ***p*<0.01, Student t-test (n=4-5). **c)** Mitochondrial superoxide production in Tetramer⁺ CD8⁺ T cells by MitoSox. Spleens from MCMV-immunized WT and T-*Atg7*^{-/-} mice were stained with MitoSox Red on day 15 post-infection and analyzed by flow cytometry. Bar graph depicts mean fluorescence intensity on m45-tetramer⁺ CD8⁺ T cells and is representative of three independent experiments. ***p*<0.01, by Student t-test (n=4-5). **d)** GLUT-1 expression. Tetramer⁺ CD8⁺ T cells from MCMV-immunized WT and T-*Atg7*^{-/-} mice were stained with the GFP-tagged HTLV receptor binding domain (eGFP-H_{RBD}), that binds GLUT-1, at the time points indicated. As a control, GLUT-1 was also measured on CD44^{lo} CD8⁺ T cells from unimmunized mice (naïve). Bar graph shows the percentage of cells expressing GLUT-1. **p*<0.05, as determined by Mann-Whitney U-test (n=4-5). All values are mean ± s.e.m.

Figure 15.

a) MCMV-immunized WT and T-*Atg7*^{-/-} mice were assessed for Bcl-2 expression in splenic m45-specific CD8⁺ T cells on day 9 and 22 post-infection. As a control, Bcl-2 was measured in CD44^{lo} CD8⁺ T cells from unimmunized mice (naïve). Quantification shows Bcl-2 mean fluorescence intensity. Statistics – Student's t-

test (n=4-5). **b)** GLUT-1 antibody staining on day 9 and day 22 post-infection in MCMV-immunized WT and T-*Atg7*^{-/-} mice. As a control, GLUT-1 was assessed in CD44^{lo} CD8⁺ T cells from unimmunized mice (naïve). Bar graphs indicate the frequency of m45-tetramer⁺ CD8⁺ T cells and CD44^{lo} CD8⁺ T cells (naïve) that express GLUT-1. Statistics – Mann Whitney U-test (n=4-5).

Figure 16. *Atg7*^{-/-} memory CD8⁺ T cells mount a significantly reduced recall response to secondary immunization.

a) Recall responses to influenza. Here, WT and T-*Atg7*^{-/-} mice were split into three groups. In one, mice were immunized with 0.00032 HAU PR8 influenza and the NP-specific CD8⁺ T cell response was measured in lungs on day 24 (PR8, n=4). In another group, mice were immunized with 0.00032 HAU PR8 influenza followed by 0.32 HAU X31 influenza challenge on day 24. The recall response to this challenge was measured on day 5 post-challenge using NP-specific tetramers (PR8+X31, n=6). In the third group, mice were immunized with 0.00032 HAU PR8 influenza, followed by 0.32 HAU X31 on day 24, and 30 days later immunized for a third time with 32 HAU PR8. The recall response to this third infection was measured in the lungs on day 5 post-challenge by tetramer (PR8+X31+PR8, n=4). Quantification shows frequency of CD8⁺ T cells that are NP-specific and are representative of two independent experiments. **p*<0.05, ***p*<0.01, by Mann-Whitney U-test. **b)** The *Atg7*^{-/-} CD8⁺ T cell response to influenza vaccination. WT and T-*Atg7*^{-/-} mice were vaccinated twice, 22 days apart with 32 HAU of the live attenuated H1N1 vaccine, S-Flu. Thirty days after the last vaccination, mice were challenged with 32 HAU X31 influenza. The CD8⁺ T cell response to NP was measured in lungs on day 23 post-challenge by tetramer. As a control, unvaccinated mice were challenged with 32 HAU X31 and culled on day 4 due to weight loss and morbidity. Quantification indicates the percentage of CD8⁺ T cells in the lung that are specific for NP on day 23 post-challenge. Example dot plots are shown. Data are representative of two independent experiments. **p*<0.05 by Mann-Whitney U-test (n=4-5). **c)** *Atg7*^{-/-} CD8⁺ T cell kinetics to influenza vaccination. WT and T-*Atg7*^{-/-} mice were vaccinated with S-Flu twice, 22 days apart, and the CD8⁺ T cell response to NP was tracked over time in blood by tetramer (n=4-6). **d)** *Atg7*^{-/-} CD8⁺ T cell kinetics to influenza vaccination and challenge. WT and T-*Atg7*^{-/-} mice were vaccinated as

described in (c). Thirty days after the last vaccination, mice were challenged with 32 HAU X31 influenza. Using NP-specific tetramers, the CD8⁺ T cell response to the vaccine regime and the challenge was tracked in the blood over time (n=4-6). All values are mean $\pm$ s.e.m.

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Figure 17

a) Recall CD8⁺ T cell responses in WT and T-*Atg7*^{-/-} mice. Mice were immunized once with PR8 influenza (n=4); twice with PR8+X31 influenza (n=6); or three times with PR8+X31+PR8 (n=4). Absolute counts of CD8⁺ NP-tetramer⁺ T cells were determined in the lungs at either day 24 (PR8), or day 5 post-challenge (PR8+X31 & PR8+X31+PR8). All values are mean $\pm$ s.e.m.

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Figure 18. Boosting autophagy restores CD8⁺ T cell responses to vaccination in elderly mice.

a) Autophagy gene expression in CD8⁺ T cells from young and elderly mice. CD44^{lo} and CD44^{hi} CD8⁺ T cells were purified from 6 week old and 2 year old mice using fluorescent activated cell sorting. mRNA was extracted and the expression of essential autophagy genes was measured by q-PCR. Shown is the fold change in expression in CD8⁺ T cells from old mice relative to expression in young mice (normalized to *gapdh* and *hprt*). **b)** LC3 Spot count in CD8⁺ T cells from young and old mice. Splenic CD8⁺ T cells from 8 week old and 2 year old mice were treated with an autophagy flux inhibitor for 2 hours, as a control cells were left untreated. LC3 spot count was determined on CD8⁺ CD44^{hi} T cells using ImageStream. Representative images are shown (x60 magnification).

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c) Quantification for images shown in (b). **d)** Human T cell line Jurkat was incubated with 100 μ M spermidine for 2, 4, or 6 hours or left untreated followed by whole protein extraction for LC3 Western Blot (upper panel). In the lower panel, Jurkat cells were incubated either with no spermidine (control), 100 μ M, or 500 μ M spermidine for 6 hours and then Western blotted for LC3. GAPDH was used as loading control for all Western blots. **e)** Jurkat cells were treated with 1 mM DFMO or 1 mM DFMO with 1 μ M spermidine for 48 hours or left untreated (control). In the final 6 hours of incubation, all cells were treated with 10nM bafilomycin A1 and LC3-I to LC3-II conversion was assessed by Western Blot. **f)**

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Jurkat cells were treated with 50 nM rapamycin, 100 μ M or 500 μ M spermidine for 6 hours followed by detection of phosphorylated S6 (Ser235/236) by Western Blot. As a control, cells were left untreated. **g)** CD8⁺ T cell kinetics to influenza vaccination in aged mice in the presence of spermidine. Eight-week-old young WT and twenty-three month old WT and T-*Atg7*^{-/-} mice were vaccinated 22 days apart with S-Flu. Twenty-one days prior to the first vaccination, aged WT and T-*Atg7*^{-/-} mice were administered spermidine in the drinking water at a concentration of 5mM through to the experimental endpoint. As a control, twenty-three month old WT mice were administered water alone. The CD8⁺ T cell response to NP was tracked over time in the blood by tetramer (n=4-5). Y-axis depicts the frequency of CD8⁺ T cells that are specific for NP. ***p*<0.01 by Mann-Whitney U-test. **h)** CD8⁺ T cell kinetics to influenza vaccination and challenge in aged mice in the presence of spermidine. Eight-week-old young WT and T-*Atg7*^{-/-} and twenty-three month old WT and T-*Atg7*^{-/-} mice were vaccinated as described in (g). Thirty days after the last vaccination, mice were challenged with 32 HAU X31 and the CD8⁺ T cell response to challenge was measured 9 days later in the lungs by tetramer. From 21 days prior to the first vaccination, through to the experimental end point, aged WT and T-*Atg7*^{-/-} mice were administered spermidine as before. Y-axis indicated the percentage of CD8⁺ T cells that are specific for NP (n=4-5). **i)** CD8⁺ T cell response to influenza challenge in vaccinated aged mice in the presence of spermidine. Nine days post-challenge, lungs were harvested and the NP-specific CD8⁺ T cell response to influenza challenge was measured by tetramer. Example dot plots are gated on CD8⁺ T cells. Bar chart shows absolute counts of NP-specific CD8⁺ T cells in the lung per lobe. ***P*<0.01, *****P*<0.0001 by Student t-test (n=4-5). All values are mean $\pm$ s.e.m.

Figure 19

Autophagy levels by CytoID **(a)** and LC3-II staining **(b)** in antigen-specific CD8⁺ T cells from young and old mice. Eight-week-old and two year old mice were immunized with PR8 influenza. On day 10 post-infection lungs were harvested and stained with CytoID or for LC3-II to assess autophagy levels in NP-Tetramer⁺ CD8⁺ T cells by flow cytometry. **(a)** *****p*<0.0001 (n=7). **(b)** ***p*=0.0056, (n=7).

DETAILED DESCRIPTION

DEFINITIONS

5 ***Polyamine***

As used herein, "polyamine" is an aliphatic organic compound having at least two primary amine groups. Polyamines may be cyclic, branched or single chained. Polyamines may further comprise at least one secondary amine group, for
10 example 2 secondary amines. Polyamines may comprise 2-20 carbon atoms, preferably 4-10 carbon atoms. Polyamines may be synthetic polyamines, naturally-occurring polyamines or their analogues. Naturally-occurring polyamines occur naturally in cells as physiologically active mediators in cellular processes. Exemplary naturally-occurring polyamines are putrescine, cadaverine,
15 spermidine and spermine.

Immunosenescence

As used herein, "immunosenescence" is a condition in which the functioning of a
20 subject's immune system is deteriorating/has deteriorated in correlation with increasing age of that subject. In particular, immunosenescence in a subject may be characterised by a refractory immune response to an immunogen. For example, the immune response to an immunogen of an older subject suffering from immunosenescence would be weaker in magnitude and/or effectiveness
25 than the immune response of a healthy younger subject.

Treatment of immunosenescence may be prophylactic and/or therapeutic, preferably therapeutic. Prophylactic treatment retards the progression of immunosenescence, or prevents it entirely. Therapeutic treatment restores the
30 function of the immune system at least partially, preferably entirely. For example, therapeutic treatment of immunosenescence may partially or wholly restore the magnitude and/or efficacy of a subject's immune response to an immunogen to the level of a healthy younger subject. The immune response may be any

(measurable) immune response as defined herein, for example a T cell immune response.

Adaptive immune response

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As used herein, an “adaptive immune response” to an immunogen is a cell-mediated and/or humoral immunogen-specific immune response mediated by at least T cells and B cells, respectively.

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T cells

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T cells, or T lymphocytes, are lymphocytes expressing a T cell receptor (TCR), for example an alpha/beta TCR or a gamma/delta TCR. A cellular immune response, mediated by T cells, is typically characterised by cytokine production and direct cytolytic activity. T cells can be broadly categorised as CD8 or CD4 T cells. CD8 T cells, or cytotoxic T cells, express CD8 glycoprotein and are MHC class I restricted. CD4 T cells express CD4 glycoprotein and are MHC class II restricted. CD4 cells may be sub-categorised as Th1, Th2, Th17 or regulatory T cells (Treg) according to the cytokines they express.

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T cells (CD4 or CD8 T cells) may be categorised as effector or memory T cells, based on their function and phenotype. A T cell immune response to immunogenic challenge may be characterised into two phases – the effector phase and the memory phase. Effector T cells are highly active but short-lived, peaking approximately 5-15 days post-challenge. Memory T cells have greater persistence, often continuing to circulate for a considerable time post-challenge. Persistent memory T cells are then able to respond rapidly to any further challenges with the same immunogen – a so-called recall response. Effector T cells in mice and humans are typically characterised by the expression of CD44 and the absence of CD62L, CCR7 and CD127. Memory T cells are characterised by the expression of CD44 and may be further subcategorised into ‘effector’ and ‘central’ memory T cells depending on the expression or absence of CD62L, CCR7 and CD127. Effector memory T cells are long-lived cells that reside at the biological site of a previous infection and lack CD62L, CCR7 and CD127

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expression. Central memory T cells are long-lived cells that circulate the blood and lymphatic system and express CD62L, CCR7 and CD127.

B cells

B cells, or B lymphocytes, are lymphocytes expressing a B cell receptor (BCR). A humoral immune response, mediated by B cells, is typically characterised by production of antibodies specific for the immunogen. B cells activated by an immunogenic challenge develop into two broad classes of B cells – plasma cells and memory B cells. Plasma cells are large, short-lived B cells that produce large numbers of immunogen-specific antibodies during the effector phase of the B cell response. Memory B cells produce far fewer antibodies during the effector phase but continue to circulate for a considerable time post-challenge. Persistent memory B cells are then able to respond rapidly to any further challenges with the same immunogen.

Immunogen

By “immunogen” is meant a substance to which it is possible to elicit an immunogen-specific immune response, preferably an adaptive immune response i.e. a humoral (antibody) and/or cell-mediated (T-cell) response. An immunogen may be an antigen. An immunogen may be a protein, a lipid, a polysaccharide, a glycoprotein, a lipoprotein, a lipopolysaccharide, a nucleic acid, for example DNA or RNA, a virus, a bacterium, or any fragment of those. An immunogen may be derived from a pathogen. An immunogen may be a vaccine.

Vaccine

By “vaccine” is meant an immunogen administered to a subject for the purpose of preventing or treating a disease (i.e. a vaccine may be prophylactic or therapeutic). For example, a vaccine may comprise or consist of a nucleic acid or protein from a pathogen, wherein administration of the vaccine to a subject induces an immune response that prevents disease being caused by that pathogen.

T cell vaccine

5 A "T cell vaccine" is a vaccine in which the prophylactic or therapeutic effect of the vaccine is mediated through T cells. Most vaccines in general use are antibody or B cell vaccines, in which the prophylactic or therapeutic effect is mediated by antibodies. However, in a number of diseases, for example *Mycobacterium tuberculosis* infection ("TB"), HIV/AIDS, malaria, and many cancers, humoral immune responses have at most partial or sometimes
10 negligible effect and it is therefore desirable to induce vaccine-specific T cells.

T cell vaccines are intended to primarily induce T cell immune responses. However, T cell vaccines may also induce a humoral immune response.

Subject

As used herein, "subject" is an animal, preferably a mammal, preferably a human.

Enhancing an immune response

20 As used herein, "enhancing the immune response" of a subject is the improvement of an immune response to an immunogen in terms of any one or more of magnitude, duration and efficacy, versus the immune response to the same immunogen in untreated control subjects.

Nutraceutical preparation

25 As used herein, a "nutraceutical preparation" is a preparation derived from a food source, which is supplemented or fortified with at least one active ingredient in
30 order to provide extra health benefits, in addition to the basic nutritional value found in the food source.

DESCRIPTION

Polyamines are organic aliphatic compounds having at least two primary amine groups, optionally also having at least one, for example two secondary amines.

5 Certain polyamines occur naturally in mammalian cells. These naturally-occurring polyamines have been shown to be active in a number of cellular processes. Examples of such naturally-occurring polyamines are putrescine, cadaverine, spermidine and spermine.

10 The present inventors have identified that supplementation of a subject with exogenous polyamine results in improved immune responses to immunogens. One example of such an improved immune response is the effect of polyamine administration on immunosenescence. In *in vivo* mouse models of immunosenescence, old mice (~25 months old) have a weak adaptive immune
15 response to immunogens such as vaccines. Moreover, if old mice are administered a second dose of the same immunogen/vaccine, the memory recall immune response to the boost is notably diminished in comparison to younger, healthy adult mice (6-8 weeks old) (Example 1). However, if old, immunosenescent mice are provided with polyamine supplementation (e.g.
20 exogenous spermidine), both the peak immune response and the memory recall response are restored to at least equivalent to that of healthy, younger adult mice (Example 1). In fact, the memory immune response in treated mice following the boost is frequently better than that observed for control younger adult mice.

25 Similarly, if young, healthy mice (6-8 weeks old) are provided with polyamine supplementation (e.g. exogenous spermidine), the peak immune response to influenza infection is greatly increased compared to untreated control mice of equivalent age (Example 2).

30 It is well-known that the protection afforded by a vaccine against a disease depends largely on the strength and persistence of an adaptive memory immune response. By treating immunosenescent mice with polyamines such that the memory immune response to an immunogen such as a vaccine is enhanced, the immune response to any subsequent pathogenic challenge is correspondingly

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enhanced. This enhanced response to pathogenic challenge will lead to reduced disease, pathology and/or increased survival.

Enhancing of the adaptive immune response to an immunogen, such as a vaccine, is often achieved through the use of adjuvants or through multiple immunisations, or a combination of the two. Adjuvants in general work through activating the innate immune system in order to provide additional stimulus signals to the adaptive immune system, for example through TLR signalling. However, such signals are general and non-specific and often lead to unwanted inflammatory side-effects such as swelling, redness/erythema, pain, fever and scarring. Similarly, use of multiple immunisations or higher doses of vaccines is often undesirable as it increases manufacturing expense, reduces compliance and high doses can lead to greater unwanted side-effects.

A further advantage of administering supplemental polyamines to enhance the immune response and/or treat immunosenescence (in place, at least partially, of adjuvants and/or multiple immunisations) is that such unwanted side-effects will be reduced. Many polyamines are naturally-occurring polyamines which, unlike many adjuvants, do not activate the innate immune system. As such, there will be minimal unwanted inflammatory side-effects as a result of using polyamines to enhance a subject's immune response.

Accordingly, in a first aspect, the present invention provides a polyamine for use in the treatment of immunosenescence in a subject. In certain embodiments, immunosenescence in a subject is characterised by a refractory immune response to an immunogen. A refractory immune response to an immunogen is an immune response that is weaker in magnitude and/or efficacy than the immune response of a healthy control subject. As used herein, a "healthy control" is a young, adult subject exhibiting the expected "immune age" and having no underlying conditions. An appropriate healthy control subject would be clearly apparent to the skilled person. Treatment of immunosenescence results in an improvement in an immunosenescent subject's immune response to an immunogen compared to the equivalent immune response to the same immunogen without administration of a polyamine. This is, of course, a theoretical

consideration because the same individual cannot be both treated and not treated. However, the response following administration of the polyamine may be compared against the expected response of a healthy control and/or a control subject suffering from immunosenescence (and not receiving a polyamine). In certain embodiments, treatment of immunosenescence results in an immune response to an immunogen equal to or greater than that observed for a healthy control. In other embodiments, treatment of immunosenescence results in an immune response to an immunogen greater than that observed for a control subject suffering from immunosenescence (and not receiving a polyamine).

In certain embodiments, the refractory immune response restored and/or enhanced by the treatment of immunosenescence is an adaptive immune response. In certain such embodiments, the refractory immune response is a T cell response, preferably a memory T cell response. In certain embodiments, the T cell response is a CD8 T cell response. In certain embodiments, the T cell response is a CD4 T cell response, for example a Th1 CD4 response and/or a Th2 CD4 response and/or a Th17 CD4 response. In a preferred embodiment, the refractory immune response restored by the treatment of immunosenescence is a CD8 memory T cell response.

In another embodiment, the refractory immune response restored by the treatment of immunosenescence is a B cell response, for example a memory B cell response.

In certain embodiments, the polyamine for use in the treatment of immunosenescence in a subject is administered to the subject prior to the administration of an immunogen. In such embodiments, the polyamine is administered at least one day (24 hours) prior to the administration of an immunogen. In certain embodiments, the polyamine is administered from 1 to 200 days prior to the administration of the immunogen, optionally 1 to 110 days, optionally 1 to 90 days, , optionally 1 to 52 days, preferably 1 to 21 days prior to administration of the immunogen.

In certain embodiments, the polyamine for use in the treatment of immunosenescence in a subject is administered to the subject following the administration of an immunogen. In such embodiments, the polyamine is administered at least one day (24 hours) following the administration of an immunogen. In certain embodiments, the polyamine is administered from 1 to 200 days following the administration of the immunogen, optionally 1 to 110 days, optionally 1 to 90 days, optionally 1 to 52 days, preferably 1 to 21 days following administration of an immunogen.

In certain embodiments, the polyamine for use in the treatment of immunosenescence in a subject is administered to the subject concurrently with the administration of an immunogen. In such embodiments, the polyamine is administered within 24 hours of the administration of an immunogen. In certain embodiments, the polyamine is administered within 12 hours, optionally within 6 hours, preferably simultaneously with the administration of the immunogen. Where the route of administration is the same, the polyamine and immunogen may be co-administered in the form of a single composition.

A polyamine may be used to treat immunosenescence in a subject according to any one or more of these embodiments in combination. That is, in certain embodiments, the polyamine is administered to the subject prior to and/or concurrently with and/or following the administration of an immunogen.

In certain embodiments, the polyamine is administered at least once a day. In certain such embodiments, the polyamine is administered once a day for 1 to 200 days, optionally for 1 to 110 days, optionally for 1 to 90 days, optionally for 1 to 52 days, optionally for 1 to 21 days, optionally for 1 day.

In certain embodiments, the polyamine is administered at least once a week. The polyamine may be administered for 1 to 30 weeks, optionally 1 to 16 weeks, optionally 1 to 13 weeks, optionally for 1 to 8 weeks, optionally for 1 to 3 weeks, optionally for 1 week.

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Any one or more of these embodiments may be used in combination with those embodiments wherein the polyamine is administered prior to administration of an immunogen. That is, for example, in certain embodiments wherein the polyamine is administered prior to the administration of an immunogen, the polyamine is administered once 200 days prior to the administration of an immunogen.

Equally, in certain embodiments the polyamine is administered at least once a day for 200 days prior to administration of an immunogen. Equally, in certain embodiments, the polyamine is administered at least once a day for 21 days beginning 200 days, optionally beginning 52 days, preferably beginning 21 days prior to the administration of an immunogen.

As a further example of such combinations, in certain embodiments wherein the polyamine is administered prior to administration of an immunogen, the polyamine is administered at least once a week beginning 200 days prior to administration of an immunogen. Equally, in certain embodiments, the polyamine is administered once a week for 1-3 weeks beginning 200 days, optionally beginning 52 days, preferably beginning 21 days prior to the administration of an immunogen.

In certain preferred embodiments, the polyamine is administered to a subject at least once a day for 1 to 200 days, preferably 1 to 52 days, preferably 1 to 21 days, more preferably for 21 days prior to administration of an immunogen to the subject.

Any one or more of these embodiments may be used in combination with those embodiments wherein the polyamine is administered following administration of an immunogen. That is, for example, in certain embodiments wherein the polyamine is administered following the administration of an immunogen, the polyamine is administered once 200 days following the administration of an immunogen. Equally, in certain embodiments the polyamine is administered at least once a day for 200 days following administration of an immunogen. Equally, in certain embodiments, the polyamine is administered at least once a day for 21 days ending 200 days, optionally ending 52 days, preferably ending 21 days following the administration of an immunogen.

As a further example of such combinations, in certain embodiments wherein the polyamine is administered following administration of an immunogen, the polyamine is administered at least once a week ending 200 days following administration of an immunogen. Equally, in certain embodiments, the polyamine is administered once a week for 1-3 weeks ending 200 days, optionally ending 52 days, preferably ending 21 days prior to the administration of an immunogen.

In certain preferred embodiments, the polyamine is administered to a subject at least once a day for 1 to 200 days, preferably 1 to 100 days, preferably 1 to 52 days, for example 1 to 21 days following administration of an immunogen to the subject. In certain more preferred embodiments, the polyamine is administered to a subject at least once a day for 52 days following administration of an immunogen to the subject.

Any one or more of these embodiments may be used in combination with those embodiments wherein the polyamine is administered concurrently with administration of an immunogen. That is, in certain embodiments, wherein the polyamine is administered at least once a day for a number of days, or at least once a week for a number of weeks, the specified number of days or weeks begins, ends or spans the day on which an immunogen is administered. In certain preferred embodiments, the polyamine is administered at least once a day for 21 days prior to administration of an immunogen, is administered concurrently with administration of the immunogen, and is administered for 52 days following administration – that is, the polyamine is administered at least once a day for between 1 to 110 days (74 days) spanning the administration of the immunogen.

In certain embodiments, the immunogen may be administered more than once, for example, where the immunogen is a vaccine, as a boost. In such embodiments, the above-described schedules of administration refer to the first administration of the immunogen. In certain such embodiments, the polyamine may also be administered prior to and/or concurrently with and/or following any subsequent administration of the immunogen. The above schedules of

administration of the polyamine may also apply to subsequent administration of the immunogen.

5 In a second aspect, the present invention provides a polyamine for use in enhancing an immune response of a subject to an immunogen. In such embodiments, enhancing an immune response of a subject to an immunogen is increasing the magnitude and/or efficacy of a subject's immune response to a level greater than that observed for an untreated control subject.

10 In certain embodiments, the immune response enhanced by use of a polyamine is an adaptive immune response. In certain such embodiments, the immune response is a T cell response, preferably a memory T cell response. In certain embodiments, the T cell response is a CD8 T cell response. In certain
15 CD4 response and/or a Th2 CD4 response and/or a Th17 CD4 response. In a preferred embodiment, the enhanced immune response is a CD8 memory T cell response.

20 In another embodiment, the enhanced immune response is a B cell response, for example a memory B cell response.

25 In certain embodiments, the polyamine for use in enhancing the immune response of a subject to an immunogen is administered to the subject prior to the administration of an immunogen. In such embodiments, the polyamine is administered at least one day (24 hours) prior to the administration of an immunogen. In certain embodiments, the polyamine is administered from 1 to 200 days prior to the administration of the immunogen, optionally 1 to 110 days, optionally 1 to 90 days, optionally 1 to 52 days, preferably 1 to 21 days prior to administration of the immunogen.

30 In certain embodiments, the polyamine for use in enhancing the immune response of a subject is administered to the subject following the administration of an immunogen. In such embodiments, the polyamine is administered at least one day (24 hours) following the administration of an immunogen. In certain

embodiments, the polyamine is administered from 1 to 200 days following the administration of the immunogen, optionally 1 to 110 days, optionally 1 to 90 days, optionally 1 to 52 days, preferably 1 to 21 days following administration of an immunogen.

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In certain embodiments, the polyamine for use in enhancing the immune response of a subject is administered to the subject concurrently with the administration of an immunogen. In such embodiments, the polyamine is administered within 24 hours of the administration of an immunogen. In certain
10 embodiments, the polyamine is administered within 12 hours, optionally within 6 hours, preferably simultaneously with the administration of the immunogen.

A polyamine may be used to enhance the immune response of a subject according to any one or more of these embodiments in combination. That is, in
15 certain embodiments, the polyamine is administered to the subject prior to and/or concurrently with and/or following the administration of an immunogen.

In certain embodiments, the polyamine is administered at least once a day. In certain such embodiments, the polyamine is administered once a day for 1 to 200
20 days, optionally for 1 to 52 days, optionally for 1 to 21 days, optionally for 1 day.

In certain embodiments, the polyamine is administered at least once a week for 1 to 30 weeks, optionally 1 to 16 weeks, optionally 1 to 13 weeks, optionally for 1 to 8 weeks, optionally for 1 to 3 weeks, optionally for 1 week.

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Any one or more of these embodiments may be used in combination with those embodiments wherein the polyamine is administered prior to administration of an immunogen. That is, for example, in certain embodiments wherein the polyamine is administered prior to the administration of an immunogen, the polyamine is
30 administered once 200 days prior to the administration of an immunogen.

Equally, in certain embodiments the polyamine is administered at least once a day for 200 days prior to administration of an immunogen. Equally, in certain embodiments, the polyamine is administered at least once a day for 21 days

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beginning 200 days, optionally beginning 52 days, preferably beginning 21 days prior to the administration of an immunogen.

As a further example of such combinations, in certain embodiments wherein the polyamine is administered prior to administration of an immunogen, the polyamine is administered at least once a week beginning 200 days prior to administration of an immunogen. Equally, in certain embodiments, the polyamine is administered once a week for 1-3 weeks beginning 200 days, optionally beginning 52 days, preferably beginning 21 days prior to the administration of an immunogen.

In certain preferred embodiments, the polyamine is administered to a subject at least once a day for 1 to 200 days, preferably 1 to 52 days, preferably 1 to 21 days, more preferably for 21 days prior to administration of an immunogen to the subject.

Any one or more of these embodiments may be used in combination with those embodiments wherein the polyamine is administered following administration of an immunogen. That is, for example, in certain embodiments wherein the polyamine is administered following the administration of an immunogen, the polyamine is administered once 200 days following the administration of an immunogen. Equally, in certain embodiments the polyamine is administered at least once a day for 200 days following administration of an immunogen. Equally, in certain embodiments, the polyamine is administered at least once a day for 21 days ending 200 days, optionally ending 52 days, preferably ending 21 days following the administration of an immunogen.

As a further example of such combinations, in certain embodiments wherein the polyamine is administered following administration of an immunogen, the polyamine is administered at least once a week ending 200 days following administration of an immunogen. Equally, in certain embodiments, the polyamine is administered once a week for 1-3 weeks ending 200 days, optionally ending 52 days, preferably ending 21 days prior to the administration of an immunogen.

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In certain preferred embodiments, the polyamine is administered to a subject at least once a day for 1 to 200 days, preferably 1 to 100 days, preferably 1 to 52 days, for example 1 to 21 days. In certain more preferred embodiments, the polyamine is administered to a subject at least once a day for 52 days following administration of an immunogen to the subject.

Any one or more of these embodiments may be used in combination with those embodiments wherein the polyamine is administered concurrently with administration of an immunogen. That is, in certain embodiments, wherein the polyamine is administered at least once a day for a number of days, or at least once a week for a number of weeks, the specified number of days or weeks begins, ends or spans the day on which an immunogen is administered. In certain preferred embodiments, the polyamine is administered at least once a day for 21 days prior to administration of an immunogen, is administered concurrently with administration of the immunogen, and is administered for 52 days following administration – that is, the polyamine is administered at least once a day for between 1 to 110 days (74 days) spanning the administration of the immunogen.

In certain embodiments, the immunogen may be administered more than once, for example, where the immunogen is a vaccine, as a boost. In such embodiments, the above-described schedules of administration refer to the first administration of the immunogen. In certain such embodiments, the polyamine may also be administered prior to and/or concurrently with and/or following any subsequent administration of the immunogen. The above schedules of administration of the polyamine may thus also apply to subsequent administration of the immunogen.

In a third aspect, the invention provides a method of enhancing the immune response of a subject to an immunogen, the method comprising:

- (i) administering a polyamine to the subject; and
- (ii) administering the immunogen to the subject.

According to this aspect of the invention, enhancing an immune response of a subject to an immunogen is increasing the magnitude and/or efficacy of a

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subject's immune response to a level greater than that observed for an untreated control subject.

5 In certain embodiments, the immune response enhanced by the method is an adaptive immune response. In certain such embodiments, the immune response is a T cell response, preferably a memory T cell response. In certain
10 embodiments, the T cell response is a CD8 T cell response. In certain embodiments, the T cell response is a CD4 T cell response, for example a Th1 CD4 response and/or a Th2 CD4 response and/or a Th17 CD4 response. In a preferred embodiment, the enhanced immune response is a CD8 memory T cell response.

15 In another embodiment, the enhanced immune response is a B cell response, for example a memory B cell response.

20 In certain embodiments, administration of a polyamine to a subject according to step (i) is prior to the administration of an immunogen to the subject in step (ii). In such embodiments, the polyamine is administered at least one day (24 hours) prior to the administration of an immunogen. In certain embodiments, the polyamine is administered from 1 to 200 days prior to the administration of the immunogen, optionally between 1 to 100 days, between 1 to 52 days, preferably between 1 to 21 days prior to administration of the immunogen.

25 In certain embodiments, administration of a polyamine to a subject according to step (i) is following the administration of an immunogen to the subject in step (ii). In such embodiments, the polyamine is administered at least one day (24 hours) following the administration of an immunogen. In certain embodiments, the polyamine is administered from 1 to 200 days following the administration of the immunogen, optionally 1 to 110 days, optionally 1 to 90 days,, optionally 1 to 52
30 days, preferably 1 to 21 days following administration of an immunogen.

In certain embodiments, administration of a polyamine to a subject according to step (i) is concurrent with the administration of an immunogen to the subject in step (ii). In such embodiments, the polyamine is administered within 24 hours of

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the administration of an immunogen. In certain embodiments, the polyamine is administered within 12 hours, optionally within 6 hours, preferably simultaneously with the administration of the immunogen.

5 A polyamine may be used to enhance the immune response of a subject according to any one or more of these embodiments in combination. That is, in certain embodiments, the polyamine is administered to the subject prior to and/or concurrently with and/or following the administration of an immunogen.

10 In certain embodiments, the polyamine is administered at least once a day. In certain such embodiments, the polyamine is administered once a day for 1 to 200 days, optionally for 1 to 52 days, optionally for 1 to 21 days, optionally for 1 day.

15 In certain embodiments, the polyamine is administered at least once a week for 1 to 30 weeks, optionally 1 to 16 weeks, optionally 1 to 13 weeks, optionally for 1 to 8 weeks, optionally for 1 to 3 weeks, optionally for 1 week.

20 Any one or more of these embodiments may be used in combination with those embodiments wherein the polyamine is administered prior to administration of an immunogen. That is, for example, in certain embodiments wherein the polyamine is administered prior to the administration of an immunogen, the polyamine is administered once 200 days prior to the administration of an immunogen. Equally, in certain embodiments the polyamine is administered at least once a day for 200 days prior to administration of an immunogen. Equally, in certain
25 embodiments, the polyamine is administered at least once a day for 21 days beginning 200 days, optionally beginning 52 days, preferably beginning 21 days prior to the administration of an immunogen.

30 As a further example of such combinations, in certain embodiments wherein the polyamine is administered prior to administration of an immunogen, the polyamine is administered at least once a week beginning 200 days prior to administration of an immunogen. Equally, in certain embodiments, the polyamine is administered once a week for 1-3 weeks beginning 200 days, optionally

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beginning 52 days, preferably beginning 21 days prior to the administration of an immunogen.

5 In certain preferred embodiments, the polyamine is administered to a subject at least once a day for 1 to 200 days, preferably 1 to 52 days, preferably 1 to 21 days, more preferably for 21 days prior to administration of an immunogen to the subject.

10 Any one or more of these embodiments may be used in combination with those embodiments wherein the polyamine is administered following administration of an immunogen. That is, for example, in certain embodiments wherein the polyamine is administered following the administration of an immunogen, the polyamine is administered once 200 days following the administration of an immunogen. Equally, in certain embodiments the polyamine is administered at
15 least once a day for 200 days following administration of an immunogen. Equally, in certain embodiments, the polyamine is administered at least once a day for 21 days ending 200 days, optionally ending 52 days, preferably ending 21 days following the administration of an immunogen.

20 As a further example of such combinations, in certain embodiments wherein the polyamine is administered following administration of an immunogen, the polyamine is administered at least once a week ending 200 days following administration of an immunogen. Equally, in certain embodiments, the polyamine is administered once a week for 1-3 weeks ending 200 days, optionally ending 52
25 days, preferably ending 21 days prior to the administration of an immunogen.

In certain preferred embodiments, the polyamine is administered to a subject at least once a day for 1 to 200 days, preferably 1 to 100 days, preferably 1 to 52 days, for example 1 to 21 days. In certain more preferred embodiments, the
30 polyamine is administered to a subject at least once a day for 52 days following administration of an immunogen to the subject.

Any one or more of these embodiments may be used in combination with those embodiments wherein the polyamine is administered concurrently with

administration of an immunogen. That is, in certain embodiments, wherein the polyamine is administered at least once a day for a number of days, or at least once a week for a number of weeks, the specified number of days or weeks begins, ends or spans the day on which an immunogen is administered. In certain preferred embodiments, the polyamine is administered at least once a day for 21 days prior to administration of an immunogen, is administered concurrently with administration of the immunogen, and is administered for 52 days following administration – that is, the polyamine is administered at least once a day for between 1 to 110 days (74 days) spanning the administration of the immunogen.

In certain embodiments, the immunogen may be administered more than once, for example, where the immunogen is a vaccine, as a boost. In such embodiments, the above-described schedules of administration refer to the first administration of the immunogen. In certain such embodiments, the polyamine may also be administered prior to and/or concurrently with and/or following any subsequent administration of the immunogen. The above referenced schedules of administration of the polyamine may also be applied to subsequent administration of the immunogen.

The following are embodiments according to the first, second and third aspects of the invention.

In certain embodiments the polyamine administered to the subject is a naturally-occurring polyamine, a synthetic polyamine, or an analogue thereof. In certain embodiments, the polyamine is putrescine, cadaverine, spermidine, or spermine, or an analogue thereof. In a preferred embodiment, the polyamine is spermidine.

In certain embodiments the polyamine is administered in solution, preferably in aqueous solution. In certain embodiments the polyamine is administered at a concentration 1-10 mM, such as 1-5 mM, optionally 5 mM.

In certain embodiments the polyamine is administered orally. In certain such embodiments, the polyamine is ingested. In certain preferred embodiments the polyamine is administered as an ingestible composition, for example in a

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beverage or foodstuff, preferably a beverage. In a preferred embodiment, the polyamine is administered dissolved in water.

5 In certain alternative embodiments, the polyamine is administered by injection. In certain such embodiments, the polyamine is administered intravenously, intramuscularly, intraperitoneally, intradermally, or subcutaneously. In certain alternative embodiments, the polyamine is administered by gavage, by bronchoscope, by nebuliser, or by suppository.

10 According to the first, second and third aspects of the invention, an immunogen is a substance which stimulates an immune response when administered to a subject with a healthy immune system.

15 The immunogen may be an antigen. In certain embodiments, the immunogen is derived from a pathogen. Pathogens from which an immunogen may be derived include viruses, bacteria, fungi, protozoans and helminths. In certain embodiments the immunogen is a protein, a polysaccharide, a nucleotide, a lipid, a lipoprotein, a lipopolysaccharide, or a glycoprotein. In certain embodiments the immunogen is a toxin.

20 In certain embodiments the immunogen is a vaccine. A vaccine may be a protein vaccine, a DNA vaccine, a bacterial vaccine or a viral vaccine. A bacterial vaccine may be a live, live-attenuated or killed bacterial vaccine. A viral vaccine may be a live, live-attenuated or killed viral vaccine. In certain embodiments the immunogen is a viral vector vaccine. In certain such embodiments, the viral vector vaccine comprises an exogenous nucleotide comprising and/or encoding an antigen from a pathogen.

25 In certain embodiments the immunogen is a T cell vaccine. In specific embodiments the immunogen is a viral T cell vaccine. In certain embodiments the immunogen is an influenza vaccine. In certain embodiments the immunogen is a live-attenuated influenza vaccine.

In certain embodiments the subject is a mammal, preferably a human.

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As will be appreciated by the skilled person, immunosenescence is most commonly exhibited in elderly individuals. Therefore, in certain embodiments of the first, second and third aspects of the invention, the subject is an elderly subject. For example, if the elderly subject is a human subject, the subject may be 60 years old or older, 65 years old or older, 70 years old or older.

Treatment of immunosenescence in a subject, for example an elderly subject, may be characterised by the subject exhibiting an improved immune response to an immunogen compared to the response exhibited by an untreated immunosenescent subject. For example, the response in a treated subject may be at least equivalent to the immune response exhibited by a subject not exhibiting immunosenescence, such as a healthy adult subject. In those embodiments wherein the subject is a human subject, a healthy adult may be a subject from 20-60 years old, optionally 25-50 years old, optionally 25-40 years old, and who is not exhibiting immunosenescence.

In a fourth aspect, the invention provides a nutraceutical preparation comprising a polyamine, wherein said preparation is suitable for the treatment of immunosenescence. In certain embodiments, the nutraceutical preparation is suitable for oral administration, preferably ingestion. Accordingly, the invention provides an ingestible nutraceutical preparation comprising a polyamine, wherein said preparation is suitable for the treatment of immunosenescence. In certain preferred embodiments the nutraceutical preparation is a beverage. In certain embodiments the nutraceutical preparation is a dairy-based preparation, for example a yoghurt. The nutraceutical preparation may additionally contain suitable flavouring and/or colouring agents to improve the appearance and/or taste of the preparation. The nutraceutical preparation may be a liquid or may be a powder such as a freeze-dried or spray-dried composition to permit reconstitution, for example with water or another consumable liquid, prior to consumption.

In certain embodiments the nutraceutical preparation comprises a polyamine in solution, preferably in aqueous solution. In certain embodiments the nutraceutical preparation comprises a polyamine in a concentration of 1-10 mM, preferably 1-5 mM, preferably 5 mM. The nutraceutical preparation may be packaged to deliver

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a single dosage unit of the polyamine. This may be a suitable volume of liquid or for powdered forms the packaging may be provided with a suitable measure together with instructions for reconstituting the preparation.

5 In a certain embodiment, the nutraceutical preparation is for use in the treatment of immunosenescence. In certain such embodiments, the nutraceutical preparation is administered at least once a day for 1 to 200 days, optionally 1 to 110 days, optionally 1 to 90 days, optionally 1 to 52 days, optionally 1 to 21 days, optionally 1 day, optionally wherein the nutraceutical is administered once.

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In certain embodiments, the nutraceutical preparation for use in the treatment of immunosenescence is administered weekly for 1 to 30 weeks, optionally 1 to 16 weeks, optionally 1 to 13 weeks, optionally 1 to 8 weeks, optionally 1 to 3 weeks, optionally 1 week.

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The present invention is not to be limited in scope by the specific embodiments described herein. Indeed, various modifications of the invention in addition to those described herein will become apparent to those skilled in the art from the foregoing description and accompanying figures. Such modifications are intended to fall within the scope of the appended claims. Moreover, all embodiments described herein are considered to be broadly applicable and combinable with

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any and all other consistent embodiments, as appropriate.

Various publications are cited herein, the disclosures of which are incorporated by reference in their entireties.

EXAMPLES

Example 1

5 The use of a polyamine in the treatment of immunosenescence was examined in an influenza vaccination and challenge model system.

Methods10 *Mice*

Six to twelve week old C57BL/6 mice were bred in specific pathogen-free conditions. Aged mice (approximately 2 years old) were purchased from a local breeder. CD4-*Atg7*^{-/-} lack the autophagy gene *Atg7* in CD4 expressing cells – primarily T cells. Because all T cells go through a CD4 expressing stage in the thymus, *Atg7* is excised in CD8⁺ T cells as well as CD4⁺ T cells. The loss of *Atg7* renders these mice autophagy-deficient in the T cell lineage.

Polyamine treatment

20 A polyamine (spermidine) was applied to the drinking water at a concentration of 5 mM from a 1M aqueous stock solution. Fresh spermidine-containing drinking water was supplied every 3-5 days.

Influenza vaccination and infection

25 Mice were immunised intranasally with an influenza vaccine at a concentration of 32 HAU in 50 µL. The vaccine in question is derived from a H1H1 influenza strain and is attenuated due to deletion of the haemagglutinin signal sequence, meaning it can efficiently infect cells but cannot replicate and spread cell to cell. For the vaccine studies (figures 1-3), mice were vaccinated on day 0 and day 22 before being challenged with 32 HAU X31 H3N2 influenza.

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Flow Cytometry

The CD8⁺ T cell response to influenza vaccination and infection in the blood and lungs was determined with a MHC class I tetramer specific for the immunodominant epitope in C57BL/6 mice, NP₃₆₆₋₃₇₄ (amino acid sequence:

ASNENMETM). This epitope is found within the conserved Influenza nucleoprotein (NP) present in both the influenza vaccine and X31 influenza. Following tetramer staining, samples were stained with anti-CD8 α and assessed on a flow cytometer.

5

Results

Figure 1

10 Aged mice exhibit immunosenescent characteristics in response to vaccination – in particular, the CD8 $^{+}$ T cell response to vaccination in old mice is significantly diminished compared to that seen in young mice. Although the initial CD8 $^{+}$ T cell response to primary vaccination is largely normal, a second 'booster' vaccination is unable to improve the frequency of circulating CD8 $^{+}$ T cells specific for Influenza Nucleoprotein (NP) (herein referred to as vaccine-specific CD8 $^{+}$ T cells) 15 in old mice as it does in young mice. Thirty days after secondary vaccination, the frequency of vaccine-specific CD8 $^{+}$ T cells is 4-fold lower in aged mice compared to young. However, oral treatment with a polyamine (spermidine) significantly enhances the CD8 $^{+}$ T cell response in aged mice to both primary vaccination and secondary vaccination compared to untreated aged mice. Thirty days after 20 secondary vaccination, polyamine-treated aged mice exhibit a 5-fold higher frequency of circulating vaccine-specific CD8 $^{+}$ T cells than non-treated controls.

The improved CD8 $^{+}$ T cell response to vaccination following spermidine is lost in aged CD4-Atg7 $^{-/-}$ mice. These mice are autophagy deficient in the T cell lineage 25 due to a T cell-specific deletion of the essential autophagy gene Atg7, suggesting spermidine may mediate its effect through autophagy.

Figure 2

30 The CD8 $^{+}$ T cell response in the blood following influenza challenge of vaccinated mice was assessed by tetramer. Nine days post challenge, 2 year old aged mice exhibit a 50% lower frequency of NP(flu)-specific CD8 $^{+}$ T cells in the blood compared to young mice. Oral administration of a polyamine (spermidine) to aged mice prior to influenza challenge restored the frequency of flu-specific CD8 $^{+}$ T cells in aged mice to the level observed in young.

The stimulating effect of spermidine on the CD8⁺ T cell response was lost in CD4-Atg7^{-/-} mice.

5 Figure 3

10 The CD8⁺ T cell response in the lungs following influenza challenge in vaccinated mice was assessed by tetramer. Influenza challenge induces a large CD8⁺ T cell response in the lungs of young mice following vaccination (at least 60% tetramer+) (A). In comparison, the frequency of NP-specific CD8⁺ T cells in the lungs of old mice was significantly reduced – a response characteristic of immunosenescence. Oral intake of a polyamine (spermidine) in old mice increased the frequency of NP-specific CD8⁺ T cells following influenza challenge. Similarly, polyamine treatment was also able to substantially enhance the absolute number of NP-specific CD8⁺ T cells in elderly mice, beyond even
15 what was observed in the lungs of young mice (B).

This demonstrates that polyamine treatment can act to treat immunosenescence in old mice by enhancing the immune response to both vaccination and infection.

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Example 2

Methods

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Mice

Six to twelve week old C57BL/6 mice were bred in specific pathogen-free conditions.

Polyamine treatment

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A polyamine (spermidine) was applied to the drinking water at a concentration of 5 mM from a 1M aqueous stock solution. Fresh spermidine-containing drinking water was supplied every 3-5 days.

Influenza infection

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Following 8 weeks of polyamine treatment, mice were immunised with 0.001 HAU X31 Influenza intranasally in 50 µL.

Flow Cytometry

The CD8⁺ T cell response to influenza infection in the lungs was determined with a MHC class I tetramer specific for the immunodominant epitope in C57BL/6 mice, NP₃₆₆₋₃₇₄ (amino acid sequence: ASNENMETM). This epitope is found within the conserved Influenza nucleoprotein (NP) present in both the influenza vaccine and X31 influenza. Following tetramer staining, samples were stained with anti-CD8α and assessed on a flow cytometer.

Results

Figure 4

Following eight weeks of oral polyamine treatment, eight week old mice were infected with influenza and the CD8⁺ T cells response in the lungs was assessed by tetramer on day 7. Polyamine-treated mice exhibited a five-fold increase in the frequency of NP-specific CD8⁺ T cells (5%) in the lungs, compared to untreated controls (1%). This demonstrates that polyamine treatment can act to enhance the immune response to infection in young, healthy mice.

Example 3

Methods

8 week old mice were immunised intranasally with the influenza vaccine S-Flu. On day 7 the vaccine-specific CD8⁺ T cell response was assessed in the lungs. From the day of immunisation to day 7, spermidine was administered in the drinking water at a concentration of 5mM. Figure 5 shows the frequency of CD8⁺ T cells that are specific for the Influenza epitope NP₃₆₆₋₃₇₄.

Figure 5 clearly shows that continuous oral administration of spermidine following vaccination increases the immune response to a vaccine antigen.

Example 4

Methods

5 8 week old mice were immunised intranasally with the influenza vaccine S-Flu. On day 14 the vaccine specific CD8⁺ T cell response was assessed in the lungs. For one group of mice, 100 µg of spermidine was administered intraperitoneally every other day from day 0 to day 14, with the first administration performed concurrently with vaccination (Figure 6A). For another group of mice, 500 µg of spermidine was administered intraperitoneally concurrently with the vaccination. Figure 6 shows the frequency of CD8⁺ T cells that are specific for the Influenza epitope NP₃₆₆₋₃₇₄.

Figure 6A shows that parenteral administration of spermidine following immunisation results in a significantly improved immune response in healthy young adult subjects. A similar effect is observed if spermidine is administered only at the time of vaccination (Figure 6B), however the observed improvement in response is not equivalent to the response observed by administration of spermidine post-vaccination.

Example 5

In the following Example, citations refer to the references provided at the end of the Example, all of which are incorporated by reference in their entirety. Citation of a document in the following Example is not an admission that the document is relevant prior art for the purposes of this application.

During infection CD8⁺ T cells initially expand then contract, leaving a small memory pool providing long lasting immunity. While it has been described that CD8⁺ T cell memory formation becomes defective in old age, the cellular mechanism is largely unknown. Autophagy is a major cellular lysosomal degradation pathway of bulk material and levels are known to fall with age. Here, we describe a novel role for autophagy in CD8⁺ T cell memory formation. Mice lacking the autophagy gene *Atg7* in T cells failed to establish CD8⁺ T cell memory to influenza and MCMV infection. Interestingly, autophagy levels were diminished

in CD8⁺ T cells from aged mice. We could rejuvenate CD8⁺ T cell responses in elderly mice in an autophagy-dependent manner using the compound spermidine. This study suggests novel immune modulators to enhance T cell responses in the aged.

5

Upon successful clearance of a pathogen, the majority of short-lived effector T cells die and the remaining cells differentiate into a memory T cell population that provides long lasting immunity. While the cytokines, surface molecules and signaling components involved in T cell memory formation have been extensively studied, the molecular pathways supporting these cell fate decisions are poorly understood. The T memory population is (1) quiescent, (2) long-lived and actively maintained and (3) relies on mitochondrial respiration ¹. Both reduced cell cycling and longevity of this stem-cell-like population demand rigorous maintenance of the cytoplasm as debris cannot be diluted to daughter cells, reminiscent of true stem cells ^{2,3,4}. Across mammalian cell types, clearance of debris and damaged organelles such as mitochondria is typically executed via autophagy ⁵.

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A recent study showed that formation of the influenza-specific B cell memory pool requires autophagy ⁶. However, while the role of autophagy in the homeostasis of naïve T cells is well studied⁷, nothing is known about the requirement of autophagy in antigen-experienced T cells. The T cell memory pool has previously been shown to be controlled by PI3K/Akt and AMPK signaling as well as mTOR (mammalian Target Of Rapamycin) inhibition ⁸⁻¹⁰, all of which also control autophagy ¹¹. Indeed, T cell memory responses can be improved with the mTOR inhibitor rapamycin ¹⁰. Interestingly, rapamycin also rejuvenates HSCs in old mice ¹². Neither of these studies addressed autophagy as a contributing mechanism.

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Evidence for age-related declining levels of autophagy stems largely from lower organisms such as yeast, flies and worms ¹³. A recent study showed that the polyamine spermidine induces autophagy and thereby prolongs life span in model organisms ¹⁴. Our own work demonstrated decreased levels of autophagy in CD8⁺ T cells of old individuals ¹⁵.

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Here, investigating a mouse model lacking the essential autophagy gene *Atg7* specifically in T cells, we find peripheral T cell lymphopenia, leading to proliferation and an activated phenotype within the CD8⁺ T cell compartment. While *Atg7*^{-/-} T cells respond normally during the early stages of live viral challenge, a severely compromised memory CD8⁺ T cell compartment was found in response to influenza and murine cytomegalovirus (MCMV). Using bone marrow (BM) chimeras, we excluded that this is due the effects of lymphopaenia; poor CD4⁺ T cell help; exhaustion, or altered cytokine receptor expression. Moreover, autophagy was found to be highest in antigen-specific CD8⁺ T cells when compared to naïve cells. Antigen-specific *Atg7*^{-/-} CD8⁺ T cells also underwent more cell death at the time of memory formation, display compromised mitochondrial health and increased expression of the glucose receptor GLUT1, a marker for glycolysis. Furthermore, recall CD8⁺ T cell responses to repeat immunizations and vaccination protocols were greatly diminished. This being reminiscent of the human ageing immune system ¹⁶, we confirmed reduced autophagy at the transcriptional and functional level in murine T cells from old mice. Importantly, we were able to restore the CD8⁺ T cell memory response in old mice with the autophagy-inducing compound spermidine, but not in autophagy-deficient mice. Finally we found that spermidine induces autophagy independently of mTOR in T cells. Enhancing autophagy in an mTOR-independent manner may provide a safe way to improve vaccine responses in the elderly.

Results

Autophagy Controls T cell numbers in naïve T-*Atg7*^{-/-} mice

Atg7^{flox/flox} mice were bred with *CD4-Cre* mice to generate mice with defective autophagy in both CD4⁺ and CD8⁺ T lymphocytes (T-*Atg7*^{-/-}). Successful excision and thereby absence of *Atg7* mRNA and *Atg7* protein was confirmed in purified T cells (Figure 8, a and b, respectively). Using the imaging flow cytometer (ImageStream) to count LC3 puncta in CD4⁺ and CD8⁺ T cells ¹⁵, we demonstrated that functional autophagy was significantly diminished in *Atg7*^{-/-}

CD8⁺ T cells (Figure 8c, with examples of ImageStream images in right panel). In addition, using a classical technique to detect lipidated LC3, we confirmed that basal autophagy was diminished in the presence and absence of the autophagy flux inhibitor Bafilomycin A (Figure 8d).

5

Previous reports have noted a number of changes to the naïve CD8⁺ T cell compartment in the absence of autophagy, with T cell lymphopenia a consistent observation^{17, 7}. We set out to investigate if an altered naïve CD8⁺ T cell compartment exists in T-*Atg7*^{-/-} mice. We confirmed observations from previous reports using similar autophagy-deficient mouse models^{17, 18} that thymic development of CD4⁺ and CD8⁺ T cells was normal in 6 week old T-*Atg7*^{-/-} mice (Fig 7a). However, mice were lymphopenic for both CD4⁺ and CD8⁺ T cells in the lymph nodes and blood (Fig 7b and 7c). Moreover, *Atg7*^{-/-} CD8⁺ T cells exhibited an activated phenotype with increased CD44 expression (Fig 7d) and decreased CD62L expression (Fig 7e), resembling a “virtual memory” compartment¹⁹. We observed similar frequencies of central effector memory CD62L⁺CD44^{hi}, however, T-*Atg7*^{-/-} mice accumulated CD8⁺ T cells with an effector memory phenotype (CD62L⁻CD44^{hi}) (Fig 8e). Similarly, data from a mouse model in which another essential autophagy gene, *Atg5*, was deleted under the hematopoietic stem cell-specific promoter Vav, showed T cell lymphopenia and the expanded virtual memory T cell compartment (CD8⁺CD44⁺) suggesting this phenotype is not *Atg7* specific (Fig 9a and b). Next, we established that proliferation was increased in the activated CD44^{hi} CD8⁺ T cell compartment by Ki-67 staining (Fig 7f). The observed activated phenotype and increased cell turnover in *Atg7*^{-/-} CD8⁺ T cell is likely driven by homeostatic proliferation in an attempt to fill the depleted T cell niche. Indeed, the expression of the homeostatic proliferation marker CD24²⁰ was found to be significantly increased on *Atg7*^{-/-} CD8⁺ T cells (Fig 7g). To investigate whether lymphopenia drives this activated phenotype in the CD8⁺ T cell compartment, we generated 1:1 mixed bone marrow (BM) chimeras from CD45.2⁺ T-*Atg7*^{-/-} BM mixed with CD45.1⁺ wild type BM. Both BMs contributed equally to form the new hematological system (Fig 9c). *Atg7*^{-/-} CD8⁺ T cells were still diminished even in the presence of a replete T cell niche, suggesting the survival defect previously described for autophagy-deficient T cells^{17, 21} is cell-intrinsic (Fig 9d). However, the activated *Atg7*^{-/-} CD8⁺ T cell phenotype was no

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longer detected in BM chimeras as measured by the frequency of donor CD45.2⁺ CD8⁺ T cells expressing CD62L⁺ (Fig 7h) and CD44^{hi} (Fig 7i). These data indicate that the observed homeostatic proliferation and the change in surface phenotype on *Atg7*^{-/-} CD8⁺ T cells is driven by lymphopenia and is not cell-intrinsic.

5

Autophagy controls maintenance of the CD8⁺ T cell memory pool

Previous studies have shown a role for autophagy in naive T cell organelle homeostasis and survival^{18 22}. However, the importance of autophagy in CD8⁺ T cells responding to infection is unknown. To investigate this we challenged *T-Atg7*^{-/-} mice with influenza (PR8 strain) and MCMV. In *T-Atg7*^{-/-} mice, we found normal expansion of the antigen-specific effector CD8⁺ T cell (CD8⁺ T_{eff}) compartment using influenza-specific tetramers on day 10 (peak response) in the lungs (Fig 10a). However, due to the pre-existing lymphopenia observed in naïve mice, the absolute counts were diminished (Fig 11a). The CD8⁺ T_{eff} response to MCMV in the blood on day 7 was also normal (Fig 10b)²³. However, the ability of *T-Atg7*^{-/-} mice to form memory CD8⁺ T cells (CD8⁺ T_{mem}) to both influenza and MCMV is severely compromised (Fig 10c and d). Performing serial bleeding on influenza infected mice over time, we demonstrated a catastrophic collapse of the antigen-specific CD8⁺ T cell pool at day 21, resulting in a failure to retain CD8⁺ T_{mem} in *T-Atg7*^{-/-} mice (Fig 10e). Interestingly, the response to the “inflationary” epitopes m38 and IE3 from MCMV is also compromised (Fig 10f). In response to these epitopes, wildtype CD8⁺ T cells continue to expand throughout MCMV chronic infection. However, *Atg7*^{-/-} CD8⁺ T cells fail to inflate and instead undergo a dramatic contraction leading to a failure to form a MCMV-specific CD8⁺ T cell memory pool. This profound CD8⁺ T cell contraction in *T-Atg7*^{-/-} mice is also observed in response to a conventional, non-inflating MCMV epitope (m45, Fig 11b). While viral titers against influenza in the lungs of wild type and *T-Atg7*^{-/-} mice were comparable at day 3 of infection, they were significantly higher on day 6 in *T-Atg7*^{-/-} mice (Fig 10g). This is most likely due to the significantly lower absolute number of antigen-specific effector CD8⁺ T cells. As *T-Atg7*^{-/-} mice survived influenza challenge, we expect that virus is eventually cleared in all mice. In keeping with these findings, autophagy levels (CytolD) are significantly

increased in the antigen-specific CD8⁺ T cell compartment compared to naïve T cells (CD44^{lo}) in response to influenza in both spleen (Fig 10h) and lungs (Fig 10i), indicating autophagy is induced upon antigen stimulation *in vivo*.

5 **The requirement for autophagy in memory CD8⁺ T cell differentiation is cell intrinsic**

To exclude that a failure to maintain a CD8⁺ T_{mem} pool is caused by lymphopenia in T-*Atg7*^{-/-} mice, we performed viral challenge experiments in 1:1 mixed BM
 10 chimera, facilitating the observation of antigen-specific *Atg7*^{-/-} CD8⁺ T cell responses in a replete T cell environment. Even in the context of a BM chimera, *Atg7*^{-/-} CD8⁺ T cells fail to generate a CD8⁺ T_{mem} pool following influenza infection, suggesting this defect is cell intrinsic (Fig 12a). By using BM chimeras to observe antigen-specific *Atg7*^{-/-} CD8⁺ T cell responses we excluded a number of
 15 explanations as to why T-*Atg7*^{-/-} mice fail to form CD8⁺ T_{mem}: (1) lymphopenia, (2) defective CD4⁺ T cell help and (3) the excision of *Atg7* in CD4-expressing antigen-presenting cells affecting CD8⁺ T cell priming. Furthermore, a defect in the frequency of memory precursor cells might also impact upon CD8⁺ T_{mem} formation in T-*Atg7*^{-/-} mice. Mixed BM chimeras demonstrated that the frequency
 20 of short-lived effector (SLEC, CD127⁺KLRG1⁻) and memory precursor (MPEC, CD127⁺KLRG1⁺) CD8⁺ T cells was in fact normal in T-*Atg7*^{-/-} mice (Fig 12b). This suggests that autophagy is required to maintain the memory compartment rather than to establish it. We then tested whether the lack of memory response is due to exhaustion of autophagy-deficient antigen-specific CD8⁺ T cells. While in T-
 25 *Atg7*^{-/-} mice, we found a sharp increase of antigen-specific CD8⁺ T cells co-expressing the exhaustion markers PD-1⁺ and TIM-3⁺; this was not found in the mixed BM chimeras. This indicates that without lymphopenia, exhaustion is not a hallmark of *Atg7*^{-/-} CD8⁺ T cell responses and cannot explain the diminished CD8⁺ T_{mem} compartment (Fig 12c). We then sought to determine if altered expression of cytokine receptors crucial for CD8⁺ T_{mem} maintenance could explain the defective
 30 CD8⁺ T_{mem} compartment. However, both IL-7Rα (CD127) and IL-15Rα were normally expressed on *Atg7*^{-/-} MCMV-specific CD8⁺ T cells (Fig 12d and 12e).

Finally, we tested whether autophagy compromises effector/memory differentiation and measured two transcription factors typically required during this differentiation. We found that the expression of IRF4, responsible for sustaining the expansion and differentiation of CD8⁺ T_{eff}²⁴, and EOMES, a factor that promotes CD8⁺ T_{mem} formation²⁵, behaved normally over time in *Atg7*^{-/-} CD8⁺ T cells (Fig 13).

Autophagy is required to maintain healthy mitochondria and cell viability for antigen-specific CD8⁺ T cells

To understand the mechanism that leads to the failure to maintain a memory compartment in the absence of autophagy, we next checked if *Atg7*^{-/-} CD8⁺ T cells were undergoing more cell death than wild type cells. While this is well described for naïve autophagy deficient T cells⁷, it had not been analyzed in antigen-specific T cells. As expected, in wild type mice challenged with MCMV, we found highest levels of cell death among antigen-specific CD8⁺ T cells just after the peak of the effector phase on day 9, whereas in T-*Atg7*^{-/-} mice, cell death was found to increase over time (Fig 14a). This could not be explained by a change in levels of the anti-apoptotic protein Bcl2 (Fig 15a). The control of mitochondrial quality and reactive oxygen species (ROS) via mitophagy, the degradation of mitochondria, has been found to prevent cell death in T cells¹⁸. In keeping with this, mitochondrial content (Fig 14b) and mitochondrial ROS (Fig 14c) were significantly increased in *Atg7*^{-/-} antigen-specific CD8⁺ T cells. While this likely explains the increased cell death, as shown for other hematopoietic cells²¹ and T cells¹⁸, mitophagy is also thought to be essential for maintenance of healthy mitochondrial energy generation. This notion is consistent with our data in other *autophagy-deficient* hematopoietic cell types (macrophages and primary leukemic lines, submitted) demonstrating increased glycolytic enzymes by proteomic analysis, increased lactate production and decreased oxygen consumption using metabolic Seahorse measurements. Indeed, studies by Pearce *et al* showed that formation of the CD8⁺ T_{mem} pool is accompanied by a switch to mitochondrial respiration^{26 27}. However, in the physiological viral infection models used here, aiming to mimic human infection, the scarcity of

antigen-specific CD8⁺ T cells prevented us from performing these metabolic measurements. In addition, due to the loss of CD8⁺ T_{mem} in the absence of *Atg7* by day 30, any metabolic analysis was restricted to early time points when antigen-specific CD8⁺ T cells are still present in T-*Atg7*^{-/-} mice. We resorted to measurement of a surrogate marker of glycolysis, the glucose transporter GLUT-1 on antigen-specific CD8⁺ T cells by two different techniques, (a) the fluorescently labeled GLUT-1 binding domain of HTLV (human T cell leukemia virus), which specifically detects GLUT-1^{28,29} and (b) a GLUT-1 antibody staining. As expected, GLUT-1 is upregulated on CD8⁺ T_{eff} (day 9) and then downregulated on CD8⁺ T_{mem} (day 22) in wild type mice as CD8⁺ T cells switch to mitochondrial respiration (Fig 14d). However, antigen-specific *Atg7*^{-/-} CD8⁺ T_{eff} express more GLUT-1 and downregulation does not occur to the same extent at the later time points compared to WT cells (day 22) (Fig 14d). This suggests that autophagy supports the metabolic switch during the differentiation from T_{eff} to T_{mem}. This was confirmed using the GLUT-1 antibody (Fig 15b). However, treatment with the anti-diabetic drug metformin that induces mitochondrial β -oxidation metabolism in T cells²⁶ did not rescue the memory T cell compartment in this model (data not shown).

Autophagy deficiency leads to diminished recall responses

We then tested whether T-*Atg7*^{-/-} mice were able to mount a recall CD8⁺ T cell response to a secondary infection. We primed T-*Atg7*^{-/-} mice with PR8 (H1N1) followed by heterologous challenge with the X31 strain of influenza (H3N2). By challenging with a heterologous virus strain expressing different surface antigens it is possible to significantly diminish the influence of antibodies in mediating immunity to secondary infection. Thus, heterotypic immunity relies heavily on cross-reactive CD8⁺ T cell responses^{30, 31}, as opposed to homotypic immunity (for example PR8 primed, PR8 challenged) to which influenza-specific antibodies contribute^{30,32}. While the wildtype mice mounted a fast and strong recall response upon X31 challenge in PR8-primed mice (PR8+X31), this was drastically diminished in T-*Atg7*^{-/-} mice in the lungs on day 5 (Fig 16a, absolute counts in Fig 17a). Even when three live virus immunizations were administered

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(PR8 primed, challenged with X31 then with PR8), T-*Atg7*^{-/-} mice were unable to mount recall responses. Surprisingly, T-*Atg7*^{-/-} mice survived the heterotypic viral challenges despite the vastly diminished numbers of secondary CD8⁺ T_{eff}.

5 Using an established non-replicating pseudotyped influenza vaccination protocol³³, we next tested whether H1N1 vaccinated T-*Atg7*^{-/-} mice could mount secondary CD8⁺ T responses to heterotypic viral challenge. Mice were vaccinated twice, 22 days apart then challenged with X31 influenza. Large numbers of influenza NP-specific CD8⁺ T cells were detected in the lungs of
10 vaccinated wild type mice 5 days post-challenge, but not in T-*Atg7*^{-/-} mice (Fig 16b). When the CD8⁺ T cell kinetics to the vaccine regimen were followed over time in the blood in T-*Atg7*^{-/-} mice, influenza-specific CD8⁺ T cells were detected at normal frequencies to primary immunization but the secondary 'booster' vaccine was unable to induce an increase in NP-specific CD8⁺ T cell frequency
15 like it could in wildtype mice (Fig 16c). Similarly, vaccinated T-*Atg7*^{-/-} mice failed to generate significant CD8⁺ T cell responses to viral challenge in the blood (Fig 16d). As before, all mice survived the live viral challenge (data not shown). In summary, these data indicate that autophagy is required for the CD8⁺ T cell recall response to either repeated immunizations with live virus or vaccination followed
20 by live viral challenge. However, these results were not repeated in a mixed bone marrow chimera setting, making it difficult to exclude that lymphopenia, CD4⁺ T cell help or CD4-expressing antigen-presenting cells might be responsible for defective recall response in T-*Atg7*^{-/-} mice.

25 Rejuvenation of aged vaccine responses through autophagy

We reasoned that a natural setting where these findings might be applicable is the aged organism where reduced autophagy levels are found in many organs
30 and cell types¹³. Indeed, we have previously shown that autophagy levels are significantly reduced in the CD8⁺ T cells of aged healthy human donors (>65y) as compared to young donors (<30y)¹⁵. Taking results presented here so far into account, we hypothesized that these low levels may contribute to the poor CD8⁺ T_{mem} formation and inefficient influenza vaccination observed in the elderly^{34 35 36}.

We addressed whether diminished CD8⁺ T_{mem} formation can be improved in the elderly by modulating autophagy. First we showed that mRNA levels of essential autophagy genes are decreased in sorted CD8⁺ T cells from naïve old mice (2 years) as compared to young mice (8 weeks). The CD8⁺ CD44^{hi} memory compartment is particularly affected (Fig 18a). CD8⁺ T cells from old mice also showed significantly decreased autophagic flux detected by counting LC3 spots in NP-specific CD8⁺ T cells from young and old mice both in the presence and absence of an autophagy flux inhibitor (Fig 18b and 18c). This was confirmed by using two flow cytometry based autophagy detection, also in NP-specific CD8⁺ T cells (Figure 19a and b).

Next, we chose the autophagy-inducing, naturally occurring compound spermidine to modulate autophagy *in vivo* as it had been safely and effectively administered to mice previously¹⁴. We first confirmed that spermidine induces autophagy in T cells *in vitro* in a dose and time-dependent manner shown by increased levels of LC3-II (Fig 18d). Spermidine levels in the blood are known to decrease with age³⁷. To mimic this, we used the inhibitor of the natural spermidine synthesis pathway DFMO in the human Jurkat T cell line, and found that autophagy levels detected by LC3 Western Blot significantly dropped in BafA treated samples and untreated samples (Fig 18e). Autophagy levels could be rescued by the addition of very low levels of spermidine in T cells (Fig 18e). As mTOR inhibition, such as achieved with Rapamycin, is well known for its multiple side effects³⁸, we confirmed that spermidine induces autophagy in T cells in an mTOR-independent manner. Rapamycin, completely abolished the phosphorylation of S6K, a major target downstream of activated mTOR, whereas spermidine had no such effect (Fig 18f). Finally we administered spermidine to old and young mice via the drinking water at concentrations known to induce autophagy¹⁴ during the influenza vaccination protocols as in Fig 16b-d. In response to first and second vaccination, and as observed in ageing humans, old mice do not mount a robust CD8⁺ T cell response as compared to young mice. Spermidine dramatically enhanced influenza-specific CD8⁺ T cell responses in old vaccinated wildtype mice but not in old vaccinated T-*Atg7*^{-/-} mice as tracked over time in blood (Fig 18g). Similarly, the response to live influenza challenge in

the blood was improved 2-3 fold (back to levels similar to young mice) when spermidine was administered to old mice, but not in the absence of autophagy (Fig 18h). In the lungs, the number of NP-specific CD8⁺ T cells responding to influenza challenge in vaccinated old mice were improved 5-6 fold in the presence of spermidine, but had little effect on the response of aged *T-Atg7^{-/-}* mice (Fig 18i).

Discussion

Hematopoietic and immune health both decline significantly with age³⁹. With the ageing population, this has become a substantial health and socio-economic problem for the developed world. Our work sheds light on the cellular mechanisms of immune ageing and how to improve responses to vaccination in the elderly.

The naïve T cell compartment in the absence of several different essential autophagy genes has been extensively analysed⁷. These models uniformly demonstrated increased damaged mitochondria leading to cell death and peripheral lymphopenia. Here, we show how this lymphopenia drives the remaining T cells to take on an activated/memory phenotype as they proliferate in an attempt to re-fill the T cell niche, often termed “virtual memory”²². Thus, we provide a link between lymphopenia and the formation of the “virtual memory” compartment and highlight the significant changes in T cell surface phenotype that can occur in a lymphopenic environment. Interestingly, the virtual memory compartment is also a hallmark of the ageing adaptive immune system. This observed phenotype in naïve mice would have precluded a coherent analysis of antigen-activated T cells, however, the use of mixed BM chimeras crucially excluded a contribution of the skewed naïve repertoire to the CD8⁺ T_{mem} phenotype.

In this study, we showed a novel and essential link for autophagy in the formation of CD8⁺ T_{mem} to infection. The absence of autophagy also dramatically altered the ability of CD8⁺ T cells to respond to secondary infection. Interestingly, the

severely depleted CD8⁺ T cell recall response did not prevent T-*Atg7*^{-/-} mice from surviving a lethal secondary heterotypic influenza challenge. Influenza virus is typically controlled by both antibodies and cellular immunity. However, in a heterotypic challenge, the surface hemagglutinin and neuraminidase (recognized by antibodies) differ between the two heterotypic strains, while the viral internal proteins (presented by MHC class I to CD8⁺ T cells) remain the same, leaving the CD8⁺ T cells to provide protection⁴⁰. The unique protective role of CD8⁺ T cells in heterotypic immunity was described several decades ago in mice⁴⁰. The observation that the majority of CD8⁺ T cell epitopes are cross-reactive between subtypes and are located in the most conserved regions of the internal proteins (nucleoprotein and matrix protein) confirms their role. In humans, the evidence, typical for human studies, is sparse and more circumstantial. A study from 1983 by McMichael *et al* demonstrated that in experimentally infected individuals virus-specific cytotoxicity inversely correlated with the extent of virus shedding in the absence of virus-specific antibodies for the strain that was used for infection⁴¹. More recently, Lalvani *et al* found higher frequencies of pre-existing T cells to conserved epitopes in individuals who developed less severe illness in the 2009 H1N1 flu pandemic in the absence of cross-protective antibodies⁴². We would postulate therefore that the survival of the T-*Atg7*^{-/-} mice after the secondary heterotypic challenge relies on the very few effector CD8⁺ T cells generated. These data also suggest that eliciting CD8⁺ T cell memory responses in influenza vaccination is highly desirable, particularly to protect from pandemic flu.

This also raises interesting questions as to why such robust secondary T cell responses have evolved when survival can be achieved with a fraction of what is observed normally in nature. Although no CD8⁺ T_{mem} cells can be detected by conventional tetramer technology 20-30 days post-immunization in T-*Atg7*^{-/-} mice, some memory cells must remain below the detection limit at very low frequency. In addition, these *Atg7*^{-/-} CD8⁺ T cells must maintain normal functional and proliferative capacity as they are detected at early time points after secondary immunization, characteristic of true memory T cell kinetics.

To explain how autophagy affects the maintenance of CD8⁺ T_{mem} cells, we addressed two alternative hypotheses experimentally: 1) accumulation of cellular

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damage affects the long-lived CD8⁺ T_{mem} in particular and 2) autophagy is required for the switch from glycolysis to mitochondrial respiration important for the survival of CD8⁺ T_{mem} cells. Regarding the first one, we show evidence for the accumulation of mitochondria and ROS in the absence of autophagy. However, a limitation of our study is we did not test for accumulation of other organelles and protein aggregates. As for the second hypothesis, initial studies involved TRAF6 (TNFR-associated factor 6) in the regulation of CD8⁺ T_{mem} development by modulating fatty acid metabolism and thereby increasing mitochondrial respiration^{26 43}. Interestingly, TRAF6, as an E3 ubiquitin ligase, has recently been shown to stabilize essential proteins in the autophagy pathway such as Ulk1⁴⁴ and Beclin-1⁴⁵. We therefore hypothesize that TRAF6 controls metabolism via autophagy and propose three mechanisms contributing to autophagy's role in mitochondrial respiration: (1) through lipophagy, the degradation of lipids⁴⁶, autophagy provides the fatty acids that fuel the Krebs cycle with Acetyl-CoA through β -oxidation, (2) through degradation of glycolytic enzymes⁴⁷ and (3) through mitophagy, autophagy provides mitochondrial quality control that is required for functional mitochondrial ATP generation. We provide evidence for the latter mechanism here. This suggests that autophagy is important in the control of metabolic pathways in T cells. Interestingly both the accumulation of damaged mitochondria/oxidative stress as well as a bias to preferentially use glycolysis are hallmarks of the ageing cell⁴⁸.

In this study, we showed that autophagy levels are impaired in CD8⁺ T cells from aged mice. Using spermidine, we could dramatically improve the CD8⁺ T cell response to vaccination and infection in elderly mice in an autophagy-dependent manner. However, aged CD4⁺ T cells also undergo immune senescence. While inducing autophagy with spermidine in the CD8⁺ T cell compartment clearly contributes to the increased numbers of antigen-specific CD8⁺ T cells, the limitation of these experiments is that we cannot gauge the contribution of improved CD4⁺ T cell function to CD8⁺ T cell numbers.

These results offer the first cell-intrinsic explanation as to why T cell memory is defective in old age. Spermidine is a pleiotropic compound and its major function has been described in yeast as an inhibitor of histone acetyl transferases,

inducing epigenetic changes to autophagy-related gene expression¹⁴. We showed that spermidine operates independently of mTOR to induce autophagy. These findings offer the prospect of improving vaccine T cell responses in the elderly through spermidine in an mTOR-independent fashion. Based on the data presented herein, we expect that fasting or other mTOR- independent drugs such as resveratrol ^{49 50} may also be beneficial for CD8⁺ T_{mem} maintenance. However, spermidine is significantly more attractive than previously published T_{mem}-boosting compounds such as metformin ²⁶ and rapamycin ¹⁰, that come with unwelcome side effects and toxicity in humans, as spermidine would not be expected to have these effects.

Material and Methods

Mice. *CD4-Cre* mice⁵¹, were from Adeline Hajjar (University of Washington) and crossed with *Atg7^{fllox/fllox}*⁵² to obtain T-*Atg7^{-/-}* mice (*CD4-Cre⁺ Atg7^{+/-}*) on a C57BL/6 background.

5 Unless otherwise stated, all mice were 6-12 weeks of age at the start of each experiment and were age and sex matched. *CD4-Cre⁻ Atg7^{+/-}* mice were used as wildtype controls and were littermates where possible. No phenotype was observed in *CD4-Cre⁺ Atg7^{+/-}* mice. Old mice, and young control mice were purchased from Charles River, UK. C57BL/6 SJL CD45.1 mice for bone marrow chimeras were purchased from Biomedical
10 Services, Oxford. All mice were housed in Biomedical Services, Oxford and animal experiments were approved by the local ethical review committee and performed under UK project license (PPL 39/2809).

Flow Cytometry. The following antibodies were used for flow cytometry (antibody clone
15 in brackets): CD8 (Ly2) PE/PE-Cy7; CD8 (53-6.7) FITC/eF450/PE-Cy7; CD4 (GK1.5) PE/FITC/APC; TCR β (H57-597) FITC/PE/PE-Cy7/APC; CD3 (145-2C11) eF450/APC; CD62L (MEL-14) FITC/PE-Cy7; CD44 (IM7) FITC/PE-Cy7; KLRG1 (2F1) FITC/PE-Cy7; IRF4 (3E4) FITC; EOMES (Dan11mag) PE-Cy7; CD127 (A7R34) FITC/PE/eF450; Ki-67 (SolA15) eF450; CD24 (M1/69) APC; CD16/32 (93) purified Fc block; CD45.2 (104)
20 eF450; all from eBioscience (San Diego, CA, USA). PD-1 (29F.1A12) PE-Cy7; PD-1 (RMP1-30) PE; CD45.2 (104) PE-Cy7; TIM-3 (B8.2C12) PE/APC; Bcl-2 (BCL/10C4) PE; all from Biolegend (San Diego, CA, USA). Glut1 (q) PerCP; IL-15R α (BAF551) biotinylated; both from R&D Systems (Minneapolis, USA).

25 For surface staining, cells were suspended in PBS 2% FCS 5mM EDTA and stained at 4°C. Anti-CD16/32 (Fc Block, eBioscience) was generally added to antibody mix to minimize non-specific staining. LIVE/DEAD Fixable Violet Dead Cell Stain Kit (Life Technologies) was used prior to surface staining to exclude dead cells. For cytoplasmic intracellular staining (Bcl-2), cells were stained for surface antibodies then fixed in IC
30 fixation buffer before permeabilisation with perm buffer (eBioscience). Cells were then suspended in perm buffer at RT for antibody staining. For nuclear targets (IRF, EOMES, Ki-67), after surface staining cells were fixed and permeabilised with FoxP3 Fixation/Permeabilisation kit (ebioscience) before resuspension in perm buffer for intracellular staining at RT.

Absolute cell counts were performed on peripheral blood taken from the lateral tail vein of live animals, collected in heparin-coated tubes (Microvette 300, Sarstedt (Nümbrecht, Germany) to avoid coagulation. Cell counts were calculated with BD TruCount tubes (BD Bioscience, NJ, USA) according to the manufacturers instructions.

Tetramers were generated as previously described⁵³ and MHC-Class I monomers stored at -80°C. Biotinylated monomers were tetramerized with Streptavidin PE or APC at a the right concentration to achieve 1:1 ratio with biotin binding sites, and added in 1/10th volumes waiting 10 minutes between additions. Tetramerized complexes were stored at 4°C. Peptide sequences for MCMV tetramers were as follows: m45⁹⁸⁵HGIRNASFI⁹⁹³, H-2D^b-restricted; m38³¹⁶SSPPMFRV³²³, H-2K^b-restricted; IE3⁴¹⁶RALEYKNL⁴²³, H-2K^b-restricted. The peptide sequence for the influenza tetramer was as follows: NP³⁶⁶ASNENMETM³⁷⁴, H-2D^b-restricted. Cells were always stained with tetramers prior to surface antibody staining in PBS 2% FCS at 37°C for 15 minutes. Tracking tetramer-positive cells over time in the blood was performed by serially bleeding the lateral tail vein. After erythrocyte lysis with red cell lysis buffer (eBioscience), remaining white cells were stained with tetramer and surface antibodies as described above.

Autophagy detection by flow cytometry was measured using the CytolD Autophagy Detection Kit (Enzo Life Science, Exeter, UK). Splenocytes and cells isolated from the lung were first stained with CytolD according to the manufacturers' instructions, prior to tetramer and surface antibody staining. Autophagy was also measured using flow cytometry by quantifying LC3-II mean fluorescence intensity using the FlowCollect Autophagy LC3 Antibody-based Assay Kit (FCCH100171, Merk-Millipore) according to the manufacturers' instructions and always following cell surface antibody staining. Use of this kit includes a step where cytosolic LC3-I is washed from the cell, leaving only membrane bound LC3-II prior to staining.

For apoptosis detection, splenocytes were stained with m45-tetramer and surface antibody as described above, then stained with LIVE/DEAD Fixable Violet Dead Cell Stain Kit. Cells were finally stained with Annexin V PE-Cy7 (eBioscience) in Annexin V

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binding buffer at room temperature. Apoptotic cells were determined as LIVE/DEAD cell dye negative (live cells), Annexin V positive.

5 GLUT-1 was measured through binding to its ligand, the receptor binding domain (RBD) of a recombinant glycoprotein from the human T lymphotropic Virus (HTLV) fused to eGFP (H_{RBD}-eGFP)⁵⁴.

10 For mitochondrial mass analysis, cells were stained with MitoTracker Green (Life Technologies, Carlsbad, CA, USA) at 150 nM in PBS 2% FCS for 30 minutes at 37°C after tetramer and surface antibody staining. To measure mitochondrial superoxide production, MitoSOX Red (Life Technologies) was used at final concentration of 5 µM from a 5mM stock in PBS 2% FCS for 30 minutes at 37°C following tetramer and cell surface staining. All flow cytometry experiments performed on a Cyan flow cytometer (Beckman Coulter, Brea, CA, USA).

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Bone Marrow (BM) Chimera. BM cells were extracted from a single 8-week-old wildtype, T-*Atg7*^{-/-} (both CD45.2⁺), and C57BL/6 SJL mouse expressing CD45.1. After lysis erythrocytes, 3x10⁶ wildtype or T-*Atg7*^{-/-} BM cells were added to 3x10⁶ CD45.1+ BM cells (1:1 CD45.2⁺:CD45.1⁺) in a total volume of 200 µL PBS. The 1:1 BM mix was
20 injected i.v 2h after lethal irradiation (450cGy twice, 4 hours apart) to C57BL/6 SJL CD45.1+ recipients (total 6x10⁶ BM cells per mouse in 200 µL). After 8 weeks of reconstitution, mixed BM chimera were immunized with MCMV or PR8 influenza, or left unimmunized. WT and T-*Atg7*^{-/-} mice were always used as controls.

25 **Immunizations.** Mice were immunized i.v. with 1x10⁶ p.f.u MCMV in 100 µL (Smith strain ATCC: VR194). Unimmunized controls were injected with 100 µL PBS i.v. For influenza infections, mice were administered intranasally with either A/PR/8/34 (PR8, H1N1 Cambridge) influenza or X31 (H3N2) influenza at the stated dose in 50 µL viral dilution media (VDM; DMEM 0.1% BSA, 10 mM HEPES, 100U/ml Penicillin 100 µg/mL
30 streptomycin and 2mM Glutamine; all from Sigma, St Louis, MO, USA). Mice were anaesthetized with isoflurane and droplets of VDM-containing virus were applied to the nares until the total 50 µL was inhaled. Unimmunized mice were always used as controls (50 µL of VDM alone). For influenza vaccination, mice were immunized intranasally with 32 HAU pseudotyped H1N1 influenza (S-Flu)³³, using the influenza infection protocol

described above, and challenged with 32 HAU X31. Unvaccinated and fully naïve mice were used as controls in all influenza vaccine experiments. In unvaccinated mice that received X31 challenge, a combination of weight loss and clinical score was used as a humane endpoint.

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q-PCR. Cells were purified with a MoFlo cell sorter (Beckman Coulter) by their surface markers. RNA was extracted using RNeasy Kit (Qiagen, Hilden, Germany) and quantified using a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). RNA was reverse transcribed (RT) using a High Capacity RNA to cDNA kit (Applied Biosystems (AB), Foster City, CA, USA). Resulting cDNA was stored at -20°C. Real-time quantitative PCR using comparative Ct method ($\Delta\Delta C_t$) was utilized to evaluate gene expression using validated Taqman probes (AB) on a 7500 Fast Real-time PCR machine (AB). Conditions: 1. 50°C, 2 min; 2. 95°C, 10 min; 3. 95°C 15 sec; 4. 60°C 1 minute; 40 cycles of 3-4. The assay IDs for the primers of the analyzed genes are as follows:

10 Mm00504340_m1 (*Atg5*), Mm00512209_m1 (*Atg7*), Mm01264428_m1 (*Atg9*),
Mm00470550_m1 (*Atg10*), Mm00503201_m1 (*Atg12*), Mm0051717_m1 (*beclin1*),
15 Mm00458724_m1 (*Map1lc3a*), Mm01545399_m1 (*hprt*), Mm99999915_g1 (*gapdh*).

ImageStream. ImageStream (Amnis imaging flow cytometer, MA, USA) has previously been used to determine autophagic flux¹⁵. To determine LC3 spot count we stained cells for LC3-II (following cell surface staining) using the FlowCollect Autophagy LC3 Antibody-based Assay Kit (FCCH100171, Merk-Millipore) according to the manufacture's instructions. Before LC3 detection, to assess autophagic flux, cells were incubated for 2 hours with the autophagy flux inhibitor provided by the kit specified and according to the manufacturer's instructions. LC3 spot count was quantified using Ideas software (Amnis), which contains a specialised, objective spot counting feature. Images collected were all at x60 magnification.

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Western Blot. 3×10^6 Jurkat cells (human CD4⁺ T cell line) or 5×10^6 purified primary CD8⁺ T cells were lysed on ice using 100 μ L 1xNP-40 Lysis Buffer. Protein concentration in supernatant was measured using BCA Protein Assay Kit (PI-23227, Thermo Scientific) and reducing Laemmli Sample Buffer was added to make protein samples for SDS-PAGE. Proteins of 30-50 μ g per lane were separated on 16% SDS-PAGE and transferred to PVDF membrane (IPFL00010, Millipore, MA, USA). After blocking with 5%

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skim milk, the membrane was blotted using the following primary antibodies: LC3 (L8918, Sigma)(1:1000), GAPDH (MAB374, Millipore)(1:10000), pS6 (2211, Cell Signaling Technology, Danvers, MA, USA)(1:5000). For S6 blotting, membranes were blotted with anti-pS6 first, then stripped for re-blot using primary antibody against S6 (2217, Cell Signaling Technologies)(1:1000). IRDye secondary antibodies were bought from LI-COR (Lincoln, NE, USA): (926-32211, 926-68020)(1:15000). For the purification of primary CD8⁺ T cells, cells were negatively selected from splenocytes using the CD8⁺ T cell Isolation kit (130-104-075, Miltenyi) according to the manufacturer's instructions.

Influenza Viral Titers (TCID₅₀ and hemagglutination). Determination of the 50% tissue culture infective dose (TCID₅₀) was performed on MDCK-SIAT1 cells in a 96 well flat-bottom plate performed by serial dilution of lung homogenates onto 3x10⁴ MDCK-SIAT1 cells followed by incubation for 72 hours at 37°C. Virus was then detected by hemagglutination where 50 µL 1% (vol/vol) human erythrocytes (adjusted so that a 1:2 dilution gave an optical density at 600nm) was added to 50 µL of the serially diluted lung homogenates/MDCK-SIAT1 supernatant in a 96 well V-bottom plate. Hemagglutination was analyzed by the loss of teardrop formation after tilting the plate. TCID₅₀ was calculated as described by Reed and Muench⁵⁵.

Statistical Analyses. All data were presented as mean ± s.e.m. P values were determined using either a Mann-Whitney U-test or two-tailed Student's t-test using GraphPad Prism software. Significant statistical differences are indicated in the figure legends or on the graphs themselves.

In vivo spermidine treatment. Mice were administered spermidine in the drinking water 21 days prior to immunization through to the experimental endpoint. Spermidine was added at a concentration of 5 mM from a 1 M aqueous stock. Spermidine-containing drinking water was replenished every 2-3 days.

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Claims:

1. A polyamine for use in enhancing an immune response of a subject to an immunogen.
- 5 2. A polyamine for use according to claim 1, wherein the polyamine is administered to the subject prior to and/or concurrently with and/or following administration to said subject of an immunogen.
3. A polyamine for use according to claim 1 or claim 2, wherein the immune response is an adaptive T cell response, optionally a T cell immune response, optionally a memory T cell response.
- 10 4. A polyamine for use according to claim 3, wherein the immune response is a CD4 T cell immune response, for example a Th1 and/or a Th2 response, alternatively a CD8 T cell immune response.
5. A polyamine for use according to claim 1 or claim 2, wherein the immune response is a B cell immune response.
- 15 6. A polyamine for use in the treatment of immunosenescence in a subject.
7. A polyamine for use according to claim 6, wherein the polyamine is administered prior to, and/or concurrently with, and/or following the administration to the subject of an immunogen.
- 20 8. A polyamine for use according to claim 6 or claim 7, wherein immunosenescence in a subject is characterised by a refractory immune response to an immunogen.
9. A polyamine for use according to claim 8, wherein immunosenescence is characterised by a refractory adaptive immune response to an immunogen.
- 25 10. A polyamine for use according to claim 9, wherein immunosenescence is characterised by a refractory T cell immune response to an immunogen, for example a refractory memory T cell immune response.

11. A polyamine for use according to claim 10, wherein immunosenescence is characterised by a refractory CD4 T cell immune response, for example a Th1 and/or a Th2 response, alternatively by a refractory CD8 T cell immune response to an immunogen.
- 5 12. A polyamine for use according to claim 9, wherein immunosenescence is characterised by a refractory B cell immune response to an immunogen.
13. A polyamine for use according to any one of claims 8-12, wherein the immunogen administered to the subject and the immunogen to which the subject has a refractory immune response is the same immunogen.
- 10 14. A polyamine for use according to any one of claims 1-13, wherein the immunogen is a vaccine, preferably a T cell vaccine.
- 15 15. A polyamine for use according to any one of claims 1-14, wherein the polyamine is spermidine, spermine or putrescine.
16. A polyamine for use according to any one of claims 1-15, wherein the polyamine is administered to the subject in solution, preferably aqueous solution.
17. A polyamine for use according to any one of claims 1-16, wherein the polyamine is administered to the subject orally, by injection, by gavage, bronchoscope, nebuliser, or suppository.
- 20 18. A polyamine for use according to any one of claims 1-17, wherein the polyamine is administered at a concentration of 1-10 mM, preferably 1-5 mM, preferably 5 mM.
- 25 19. A polyamine for use according to anyone of claims 1-18, wherein the polyamine is administered at least once a day for 1 to 200 days, optionally 1 to 110 days, optionally 1 to 90 days, optionally for 1 to 52 days, optionally for 1 to 21 days, optionally for 1 day, or wherein spermidine is administered once.
20. A polyamine for use according to any one of claims 1-19, wherein the polyamine is administered at least once a week for 1 to 30 weeks,

optionally 1 to 16 weeks, optionally 1 to 13 weeks, optionally for 1 to 8 weeks, optionally for 1 to 3 weeks, optionally for 1 week.

21. A method of enhancing the immune response of a subject to an immunogen, the method comprising:

- 5 (i) administering a polyamine to the subject; and
- (ii) administering the immunogen to the subject.

22. The method of claim 21, wherein step (i) is performed prior to and/or concurrently with and/or following step (ii).

10 23. The method of claim 21, wherein the method enhances the adaptive immune response.

24. The method of claim 23, wherein the method enhances the T cell response, for example the memory T cell response, for example the CD8 T cell response and/or the CD4 T cell response, for example the CD4 Th1 response and/or the CD4 Th2 response.

15 25. The method of any one of claims 21-24, wherein the immunogen is a vaccine, preferably a T cell vaccine.

26. The method of any one of claims 21-25, wherein the polyamine is spermidine, spermine or putrescine.

20 27. The method of any one of claims 21-26, wherein the polyamine is administered to the subject in solution, preferably aqueous solution.

28. The method of any one of claims 21-27, wherein the polyamine is administered to the subject orally, by injection, by gavage, bronchoscope, nebuliser, or suppository.

25 29. The method of any one of claims 21-28, wherein the polyamine is administered at a concentration of 1-10 mM, preferably 1-5 mM, preferably 5 mM.

30. The method of any one of claims 21-29, wherein the polyamine is administered at least once a day for 1 to 200 days, optionally for 1 to 110

days, optionally for 1 to 90 days, optionally for 1 to 52 days, optionally for 1 to 21 days, optionally for 1 day, optionally wherein the polyamine is administered once.

5 31. The method of any one of claims 21-30, wherein the polyamine is administered at least once a week for 1 to 30 weeks, optionally for 1 to 16 weeks, optionally for 1 to 13 weeks, optionally for 1 to 8 weeks, optionally for 1 to 3 weeks, optionally for 1 week.

10 32. A polyamine for use according to any of claims 1-20 or a method according to any one of claims 21-31, wherein the subject is a mammal, for example a human.

33. A nutraceutical preparation comprising a polyamine, wherein said preparation is suitable for the treatment of immunosenescence.

34. The nutraceutical preparation according to claim 33, wherein said preparation is suitable for oral administration.

15 35. The nutraceutical preparation according to claim 34, wherein said preparation is a beverage.

36. The nutraceutical preparation according to any one of claims 33-35, wherein said preparation is a dairy-based preparation, for example a yoghurt.

20 37. The nutraceutical preparation according to any one of claims 33-36, wherein said preparation comprises the polyamine in a concentration of 1-10 mM, preferably 1-5 mM, preferably 5 mM.

38. The nutraceutical preparation according to any one of claims 33-37 for use in the treatment of immunosenescence.

25 39. The nutraceutical preparation according to any one of claims 33-37 for use according to claim 38, wherein said preparation is administered at least once a day for 1 to 200 days, optionally for 1 to 110 days, optionally for 1 to 90 days, optionally 1 to 52 days, optionally 1 to 21 days, optionally 1 day.

40. The nutraceutical preparation according to any one of claims 33-37 for use according to claim 38, wherein said preparation is administered weekly for 1 to 30 weeks, optionally 1 to 8 weeks, optionally 1 to 3 weeks, optionally 1 week, optionally wherein said preparation is administered once.

Figure 1

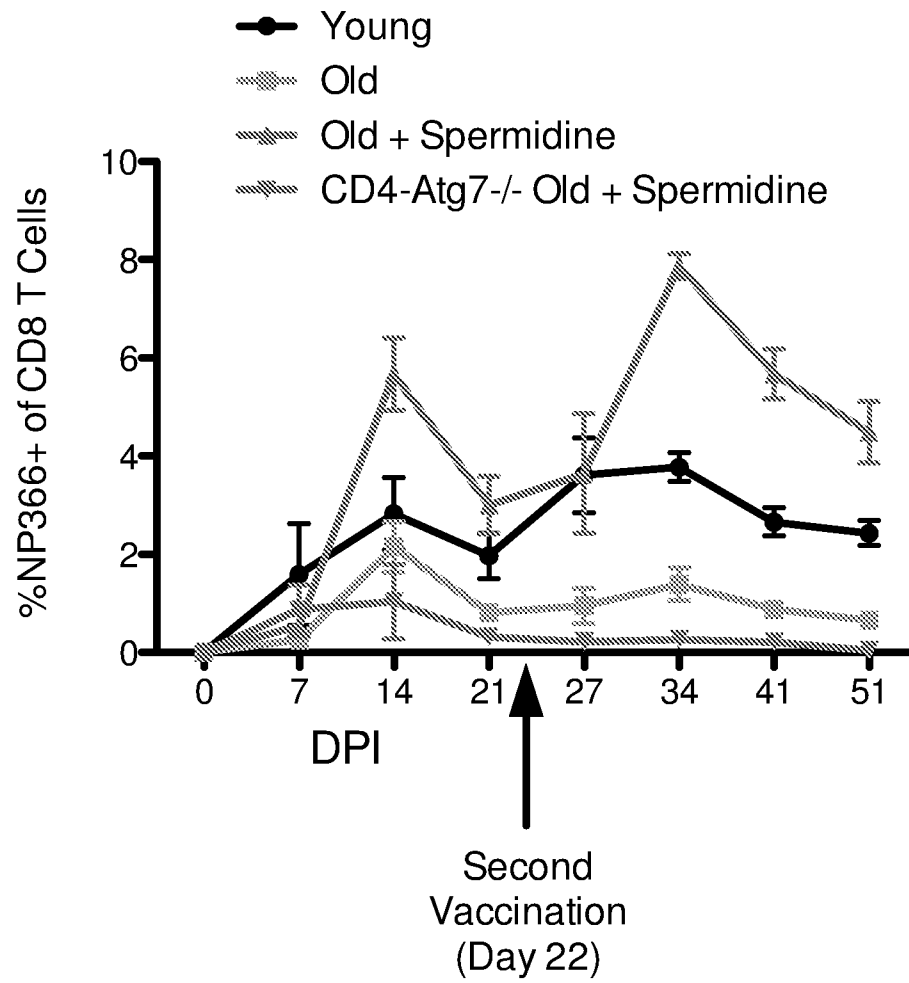


Figure 2

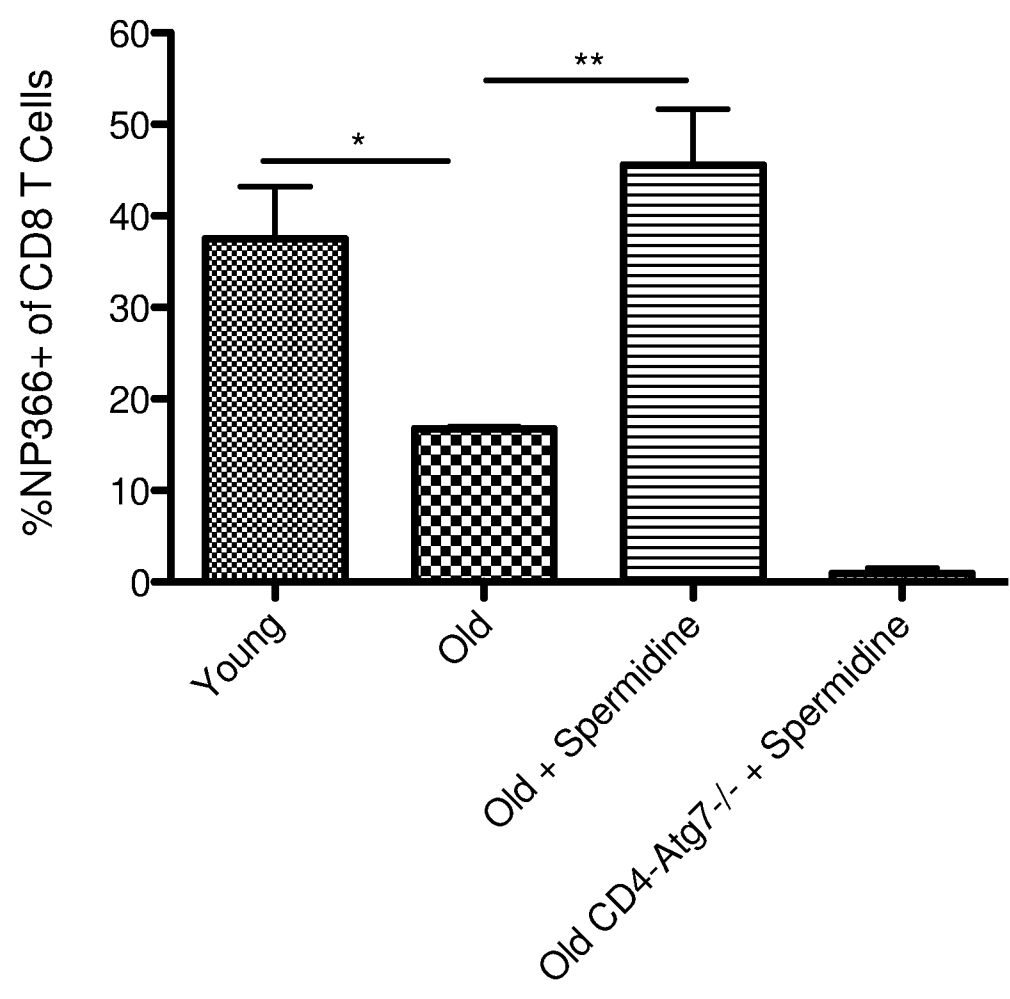
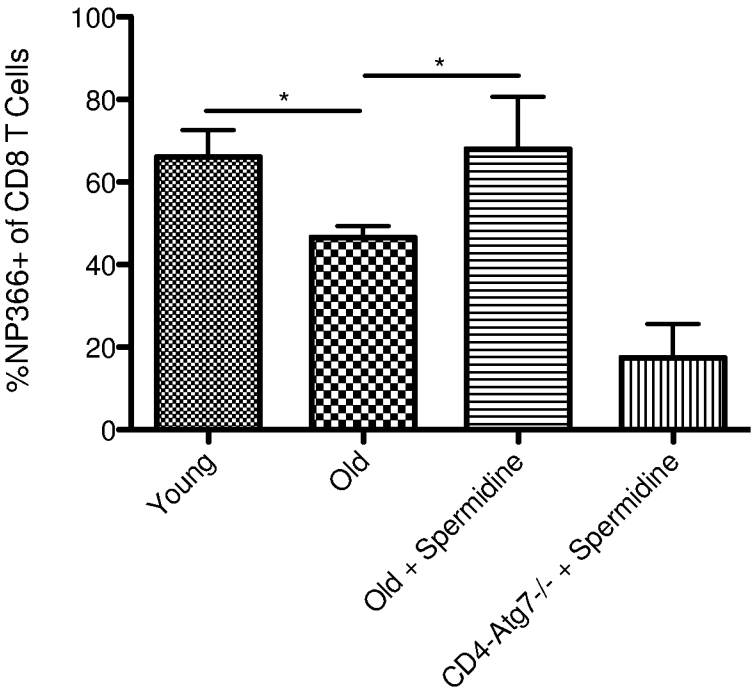


Figure 3

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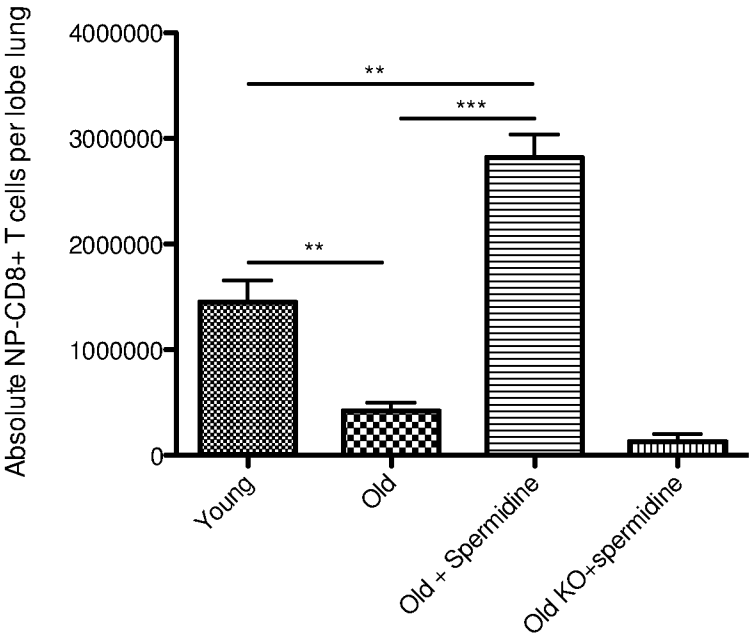


Figure 4

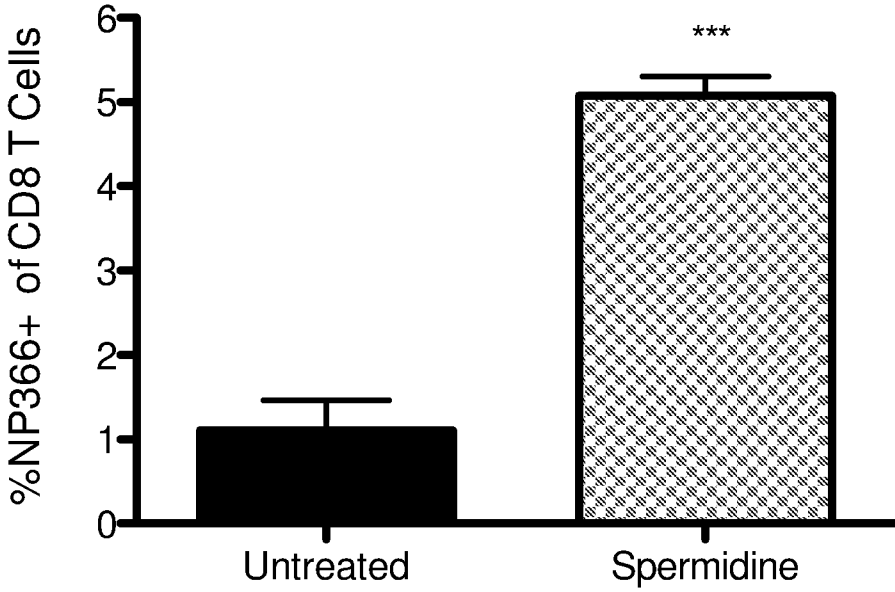


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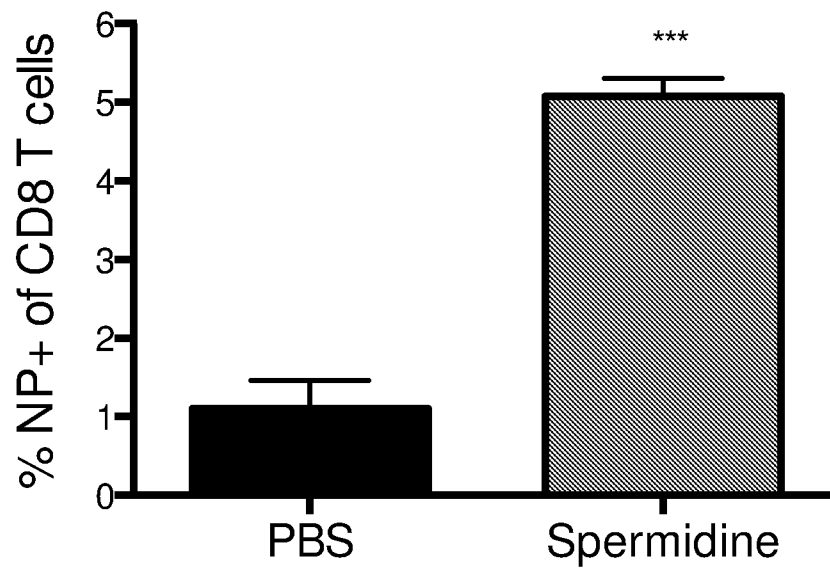


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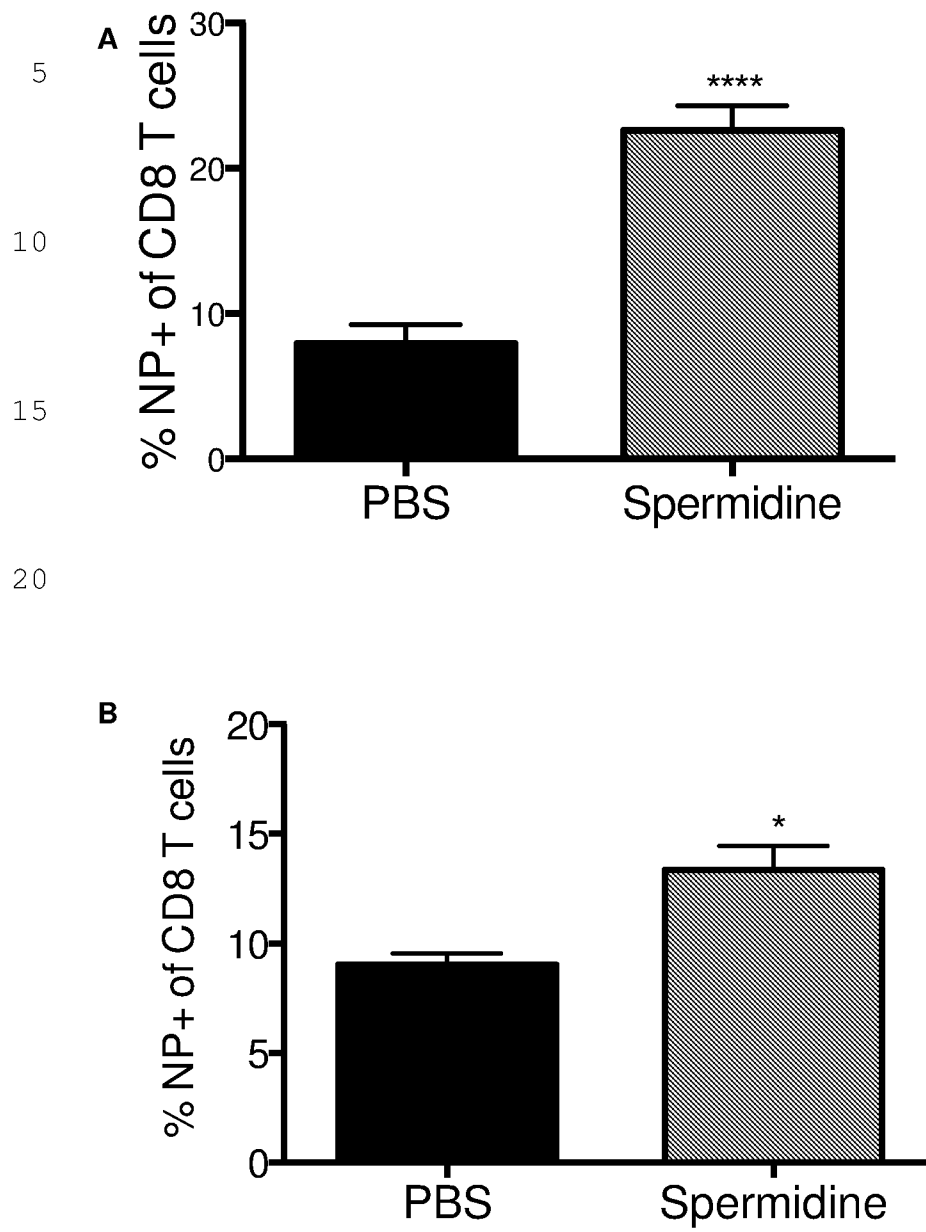


Figure 7

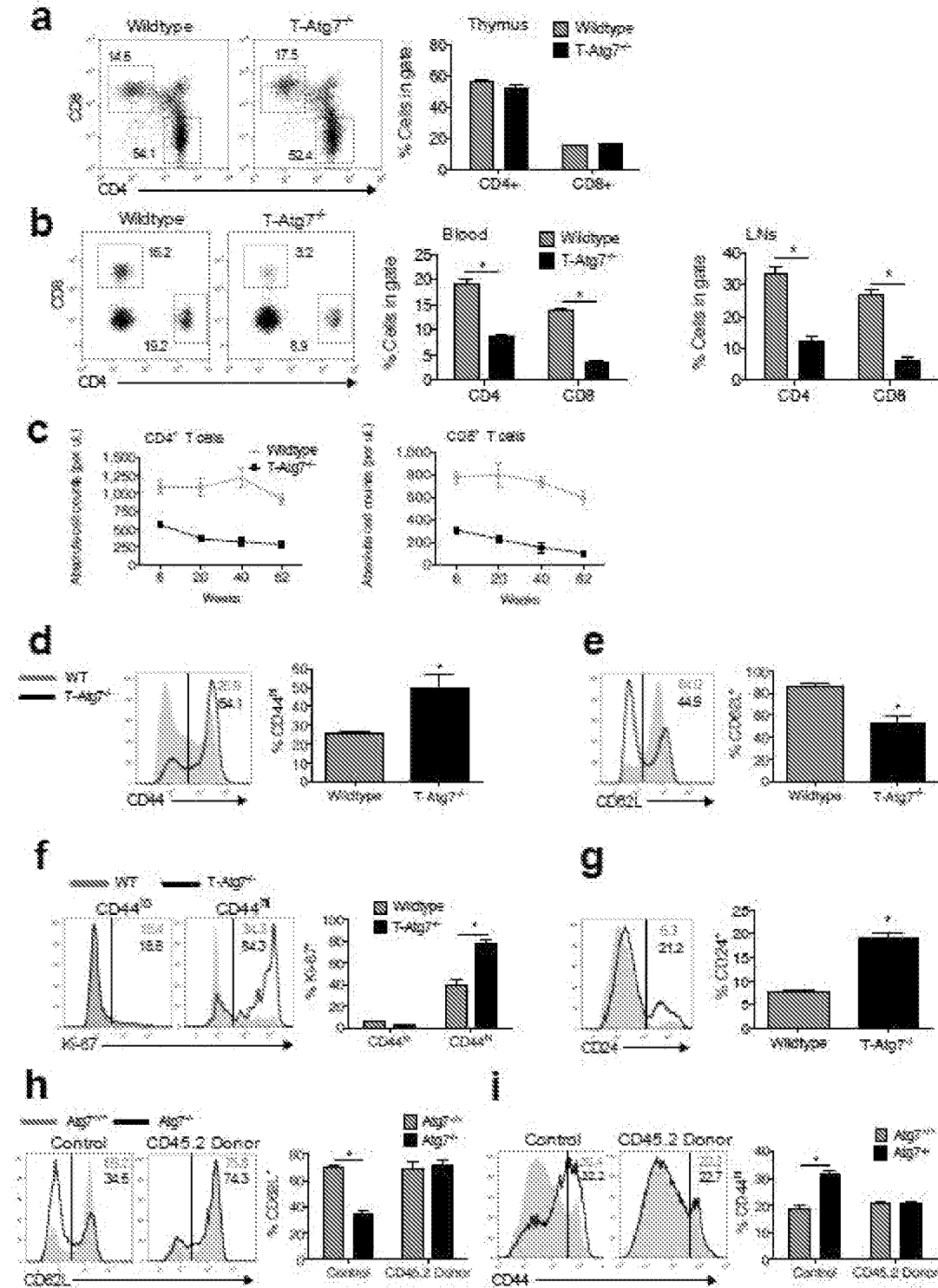


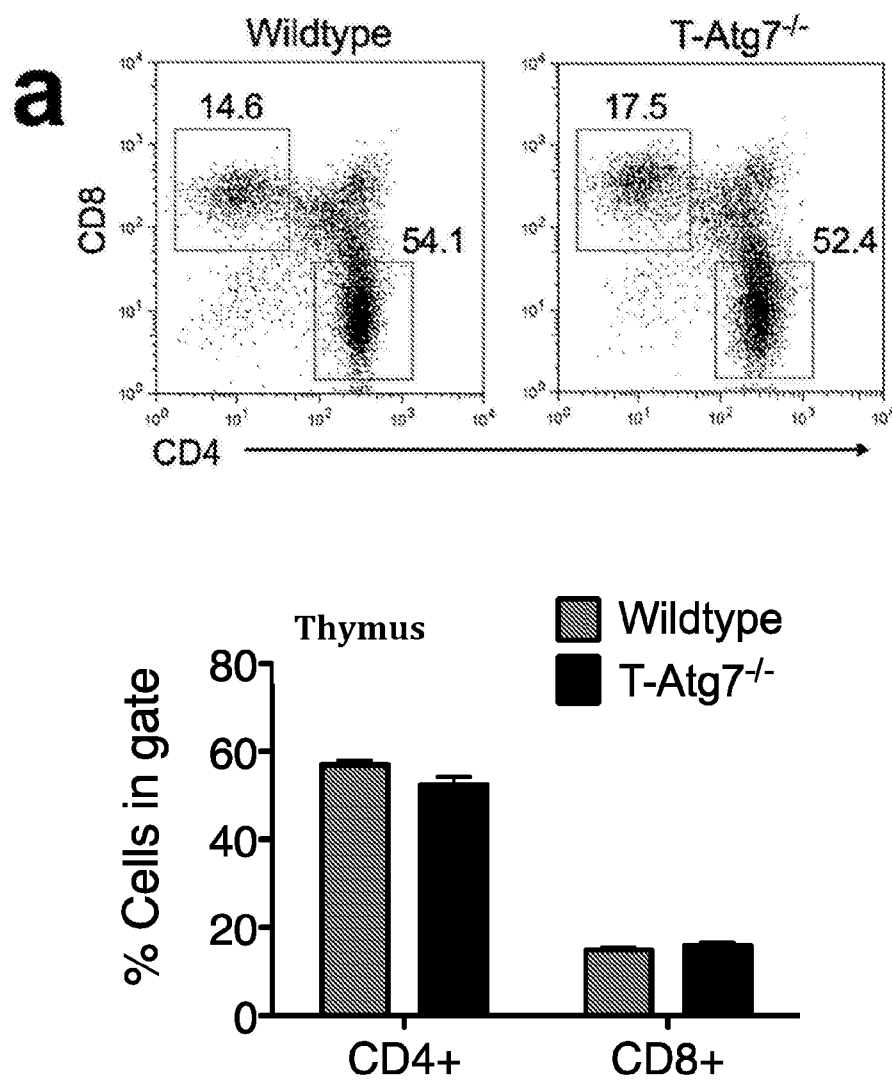
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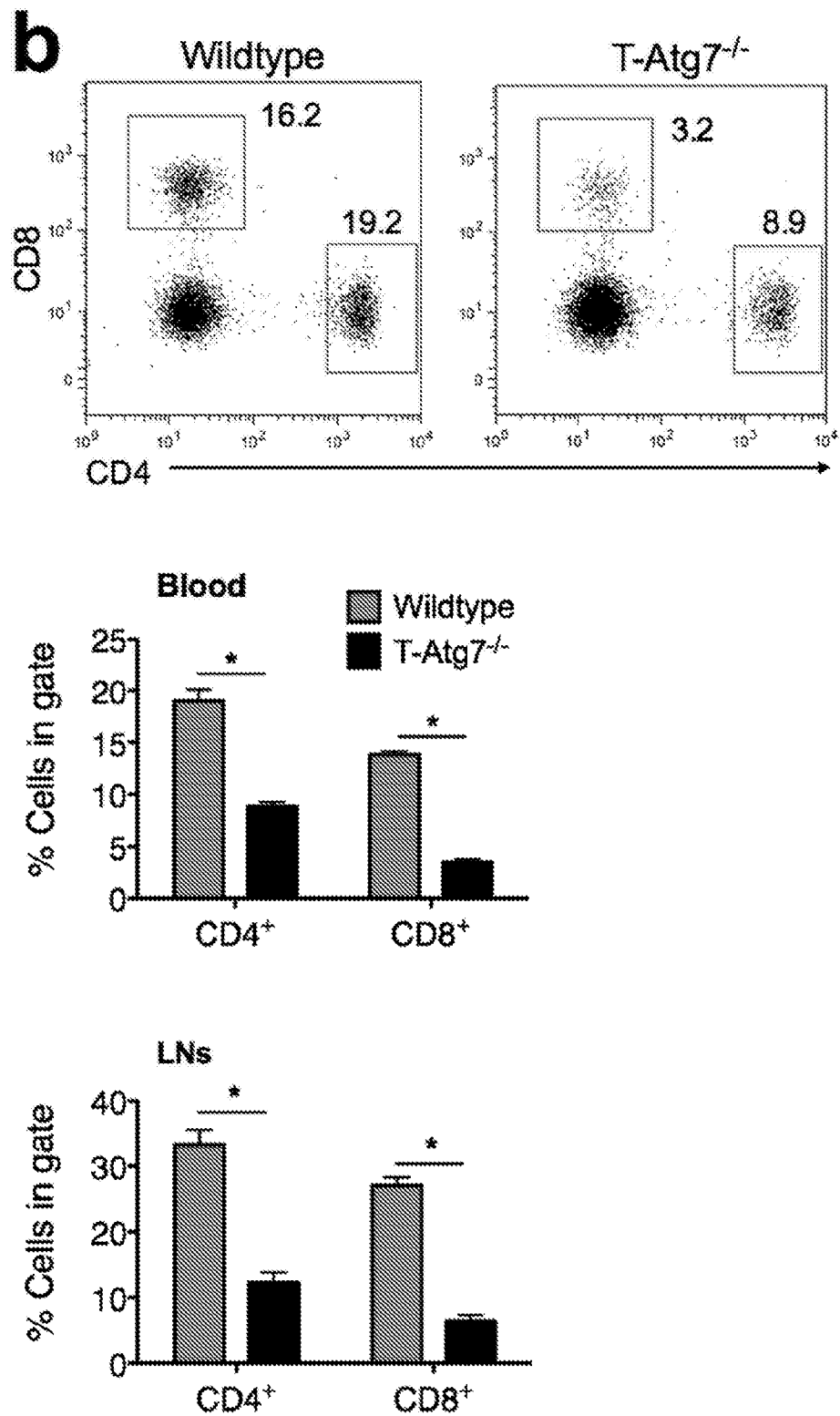
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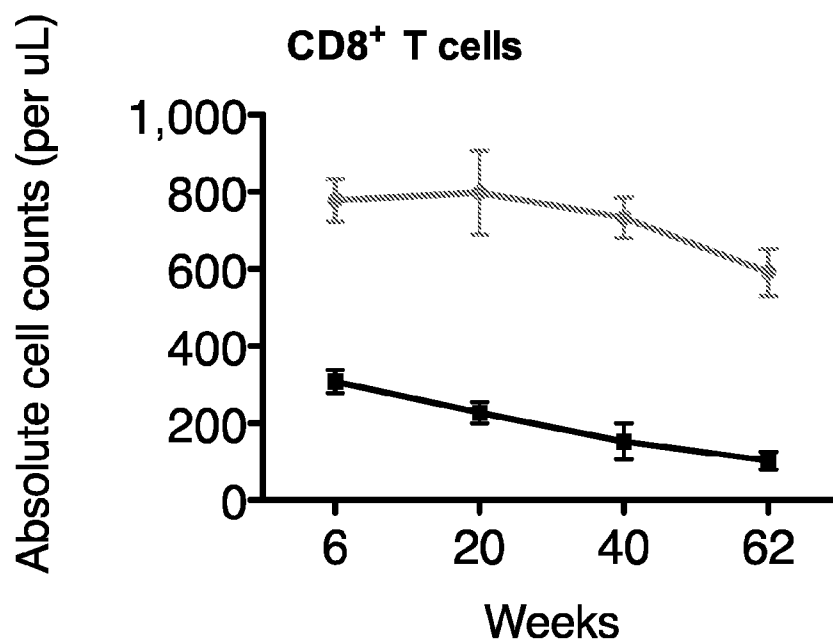
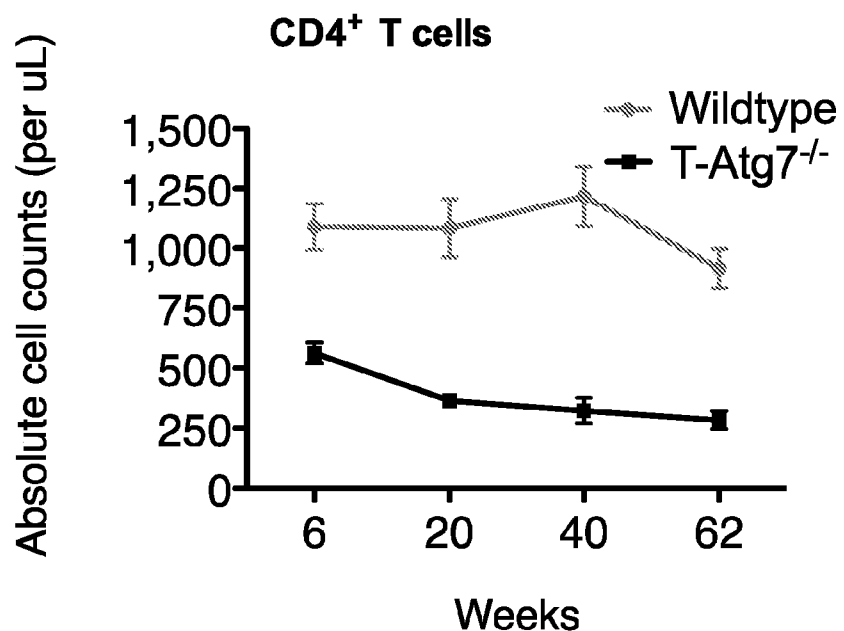
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Figure 7

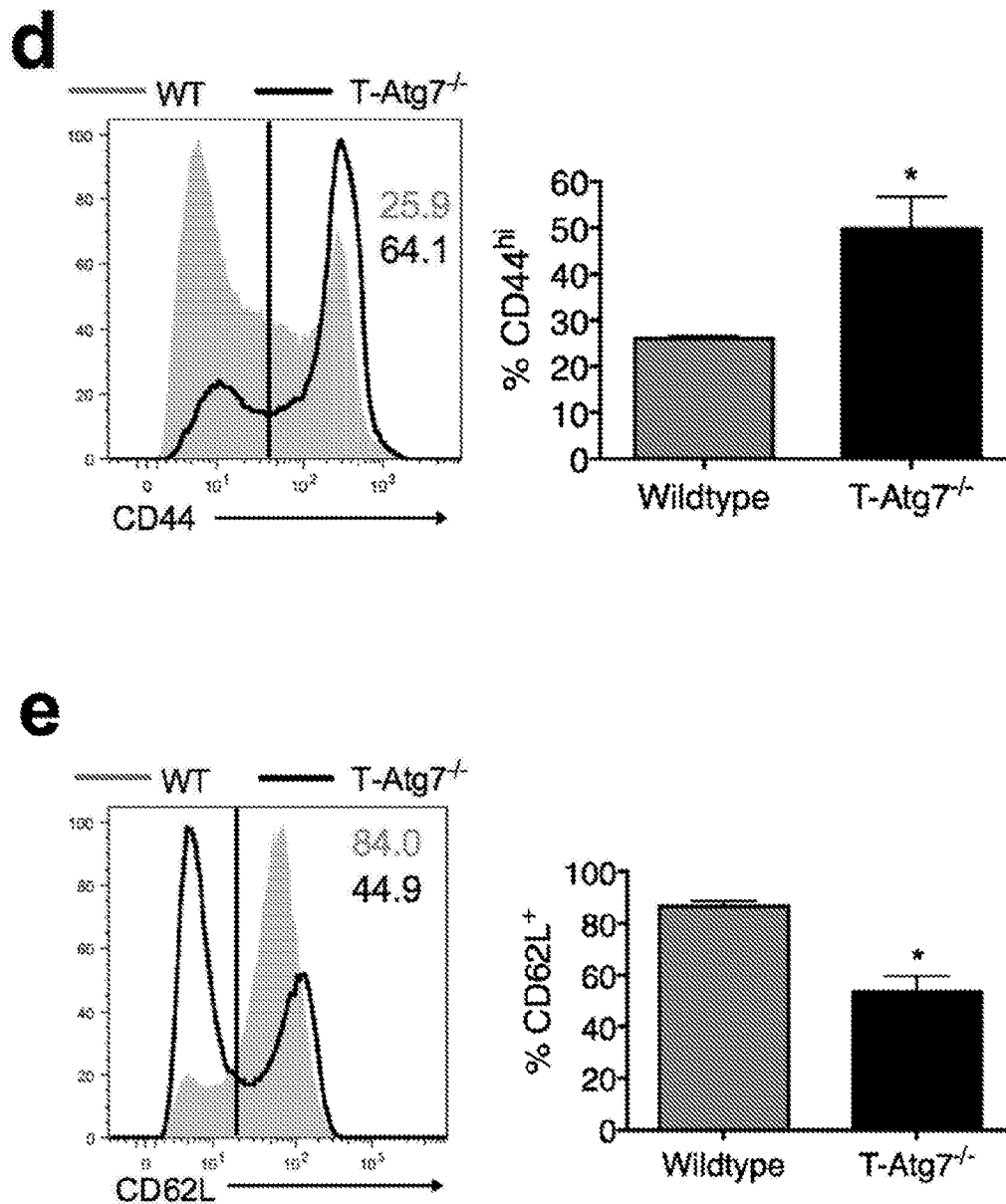


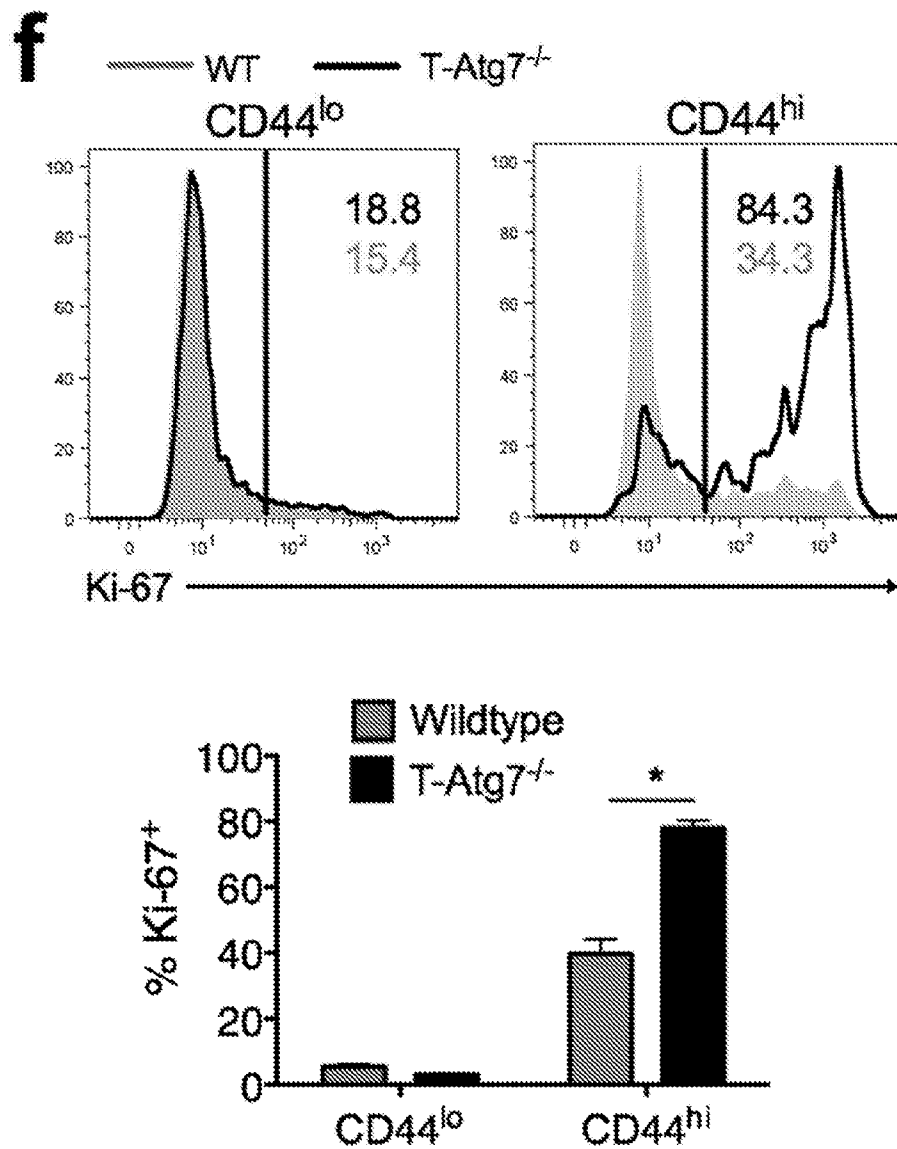
Figure 7

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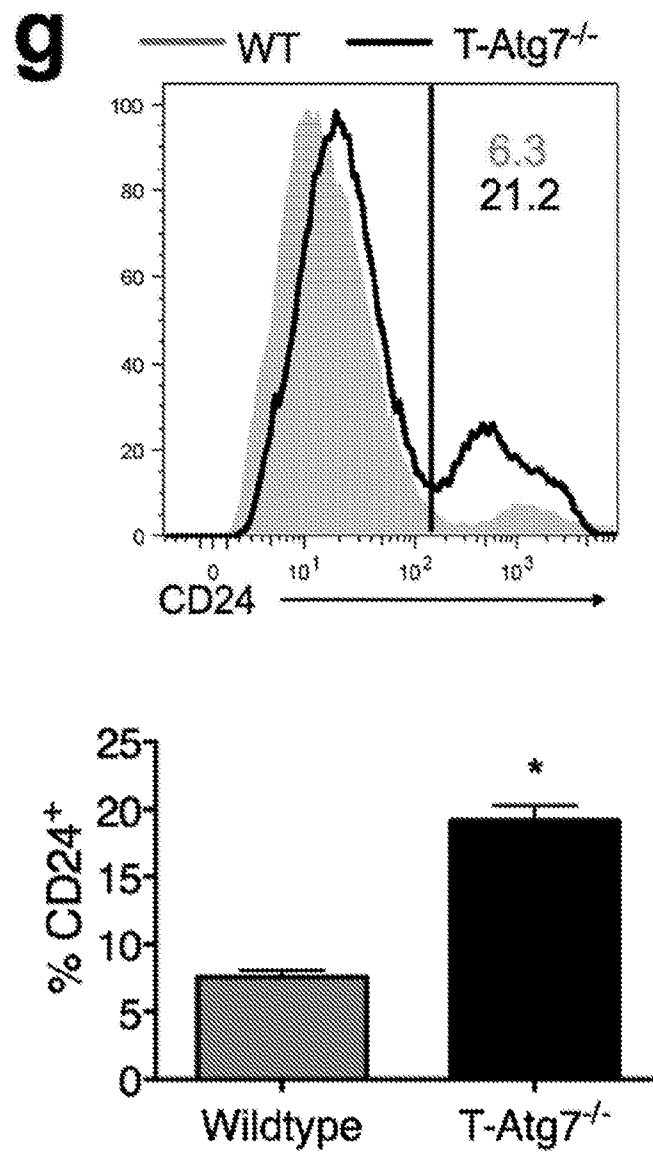


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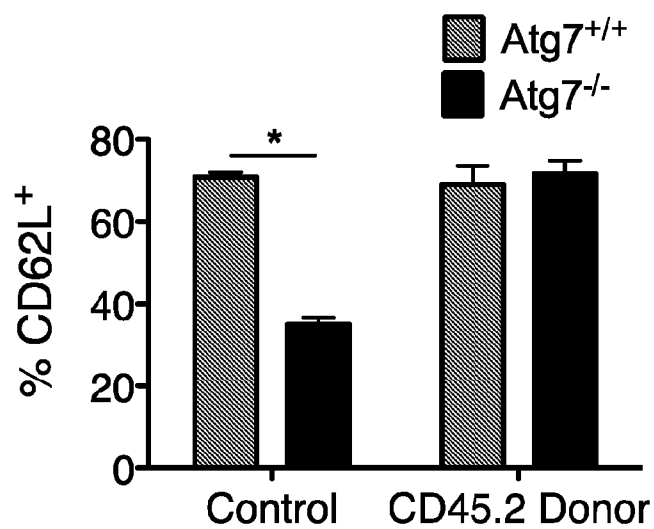
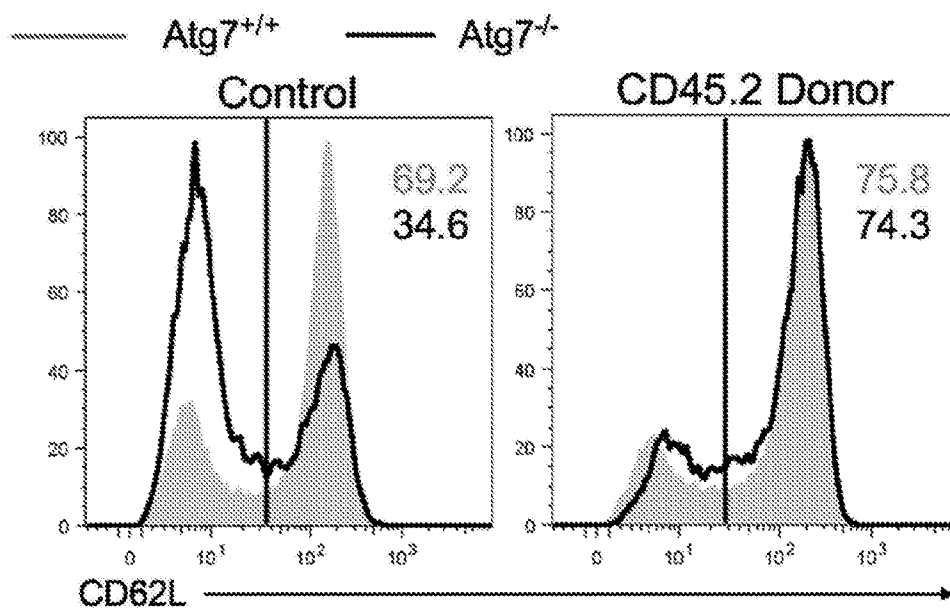
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Figure 7

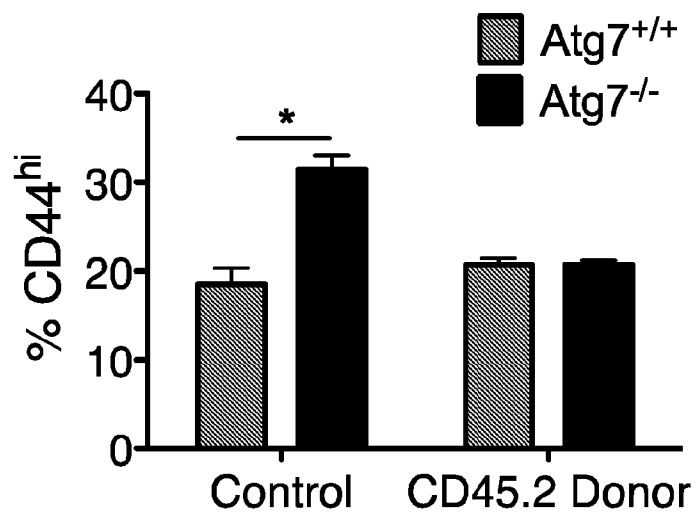
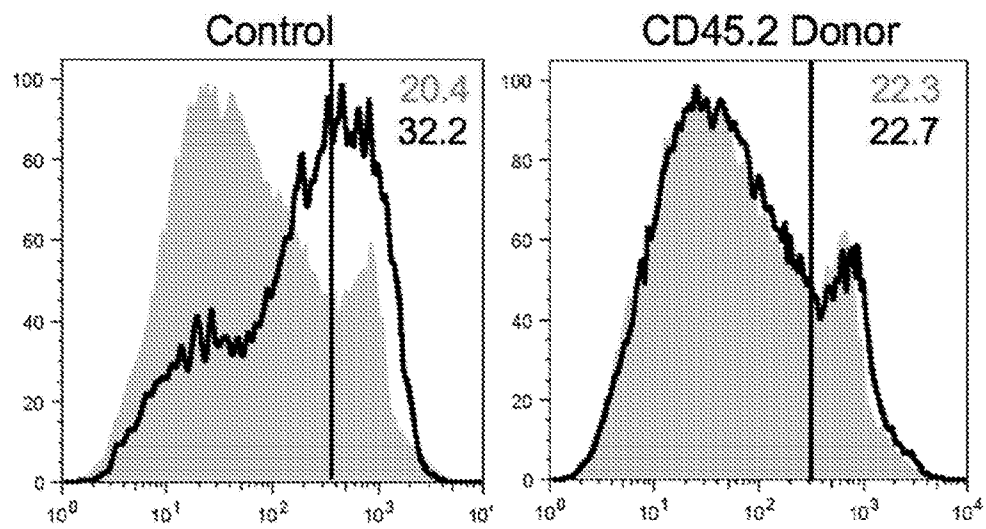
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Figure 8

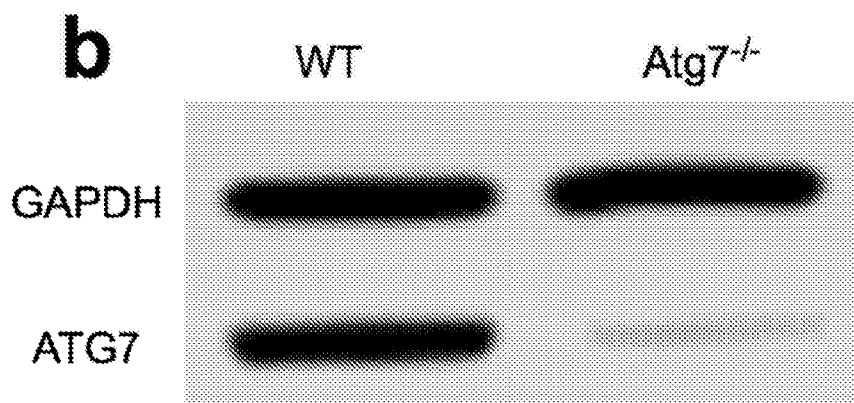
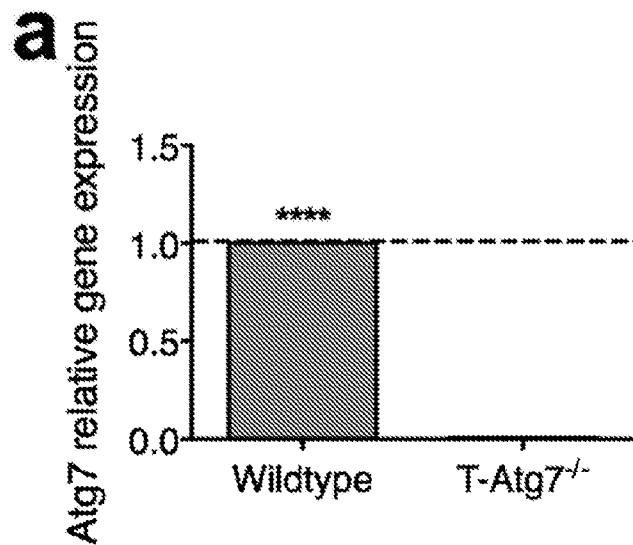


Figure 8

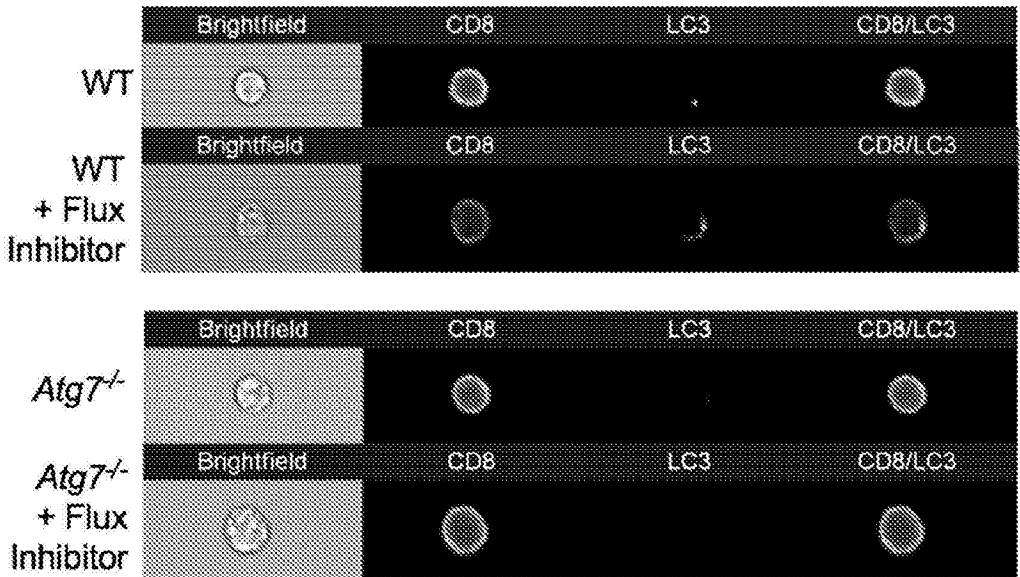
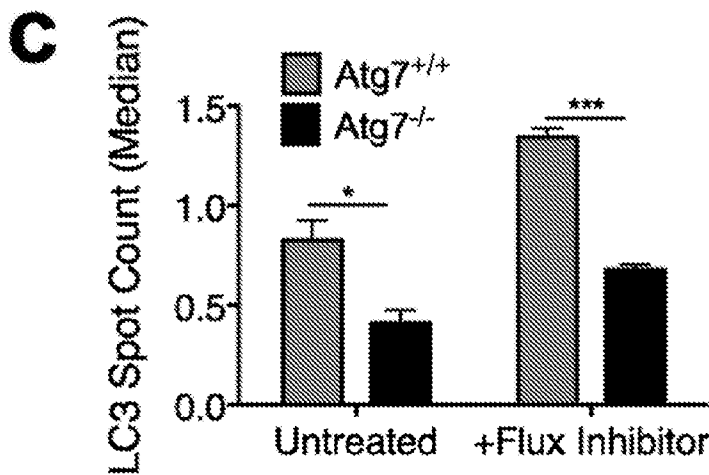


Figure 8

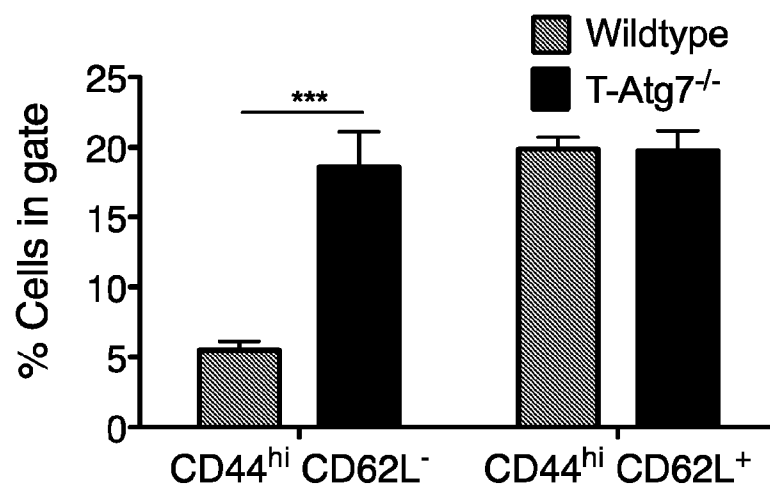
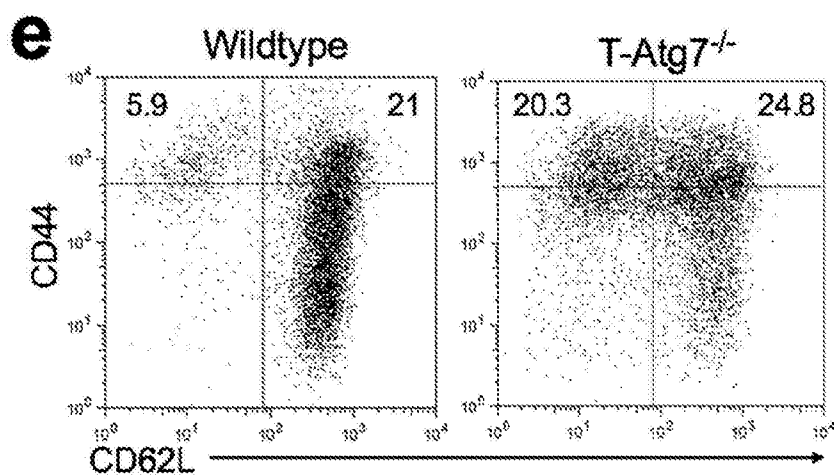
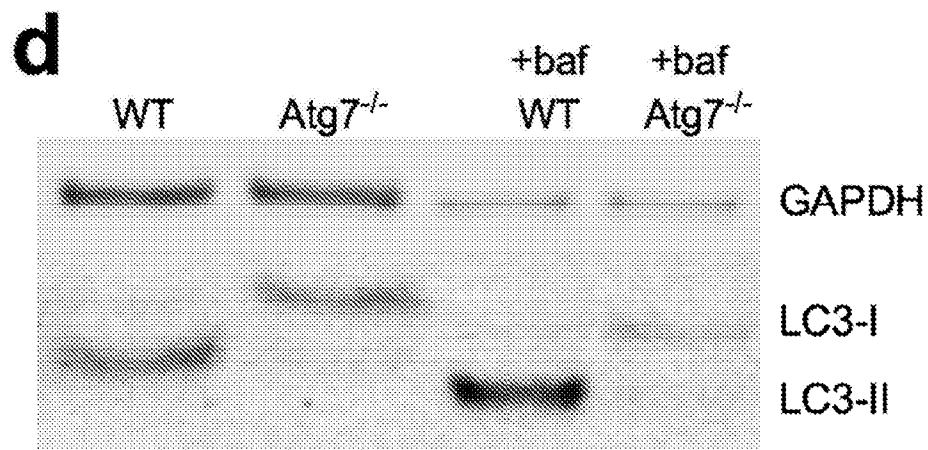


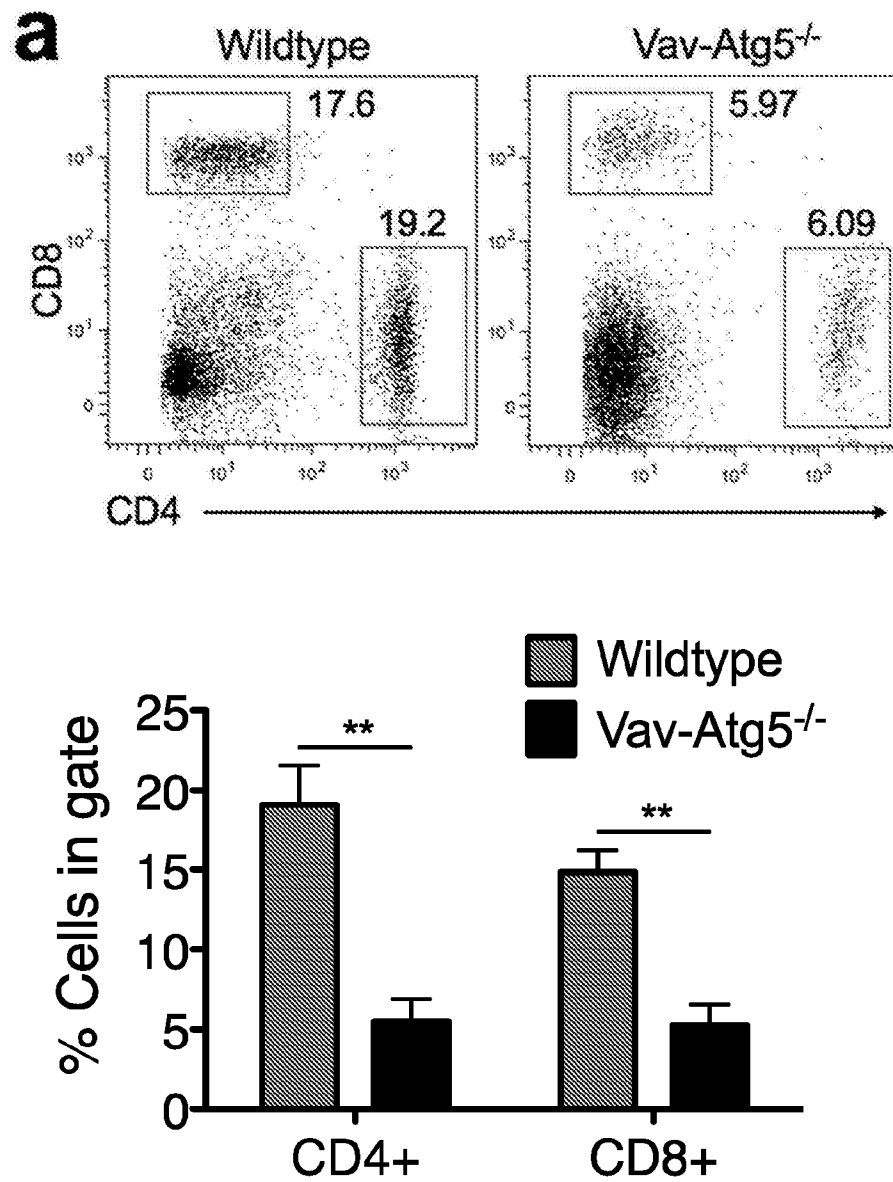
Figure 9

Figure 9

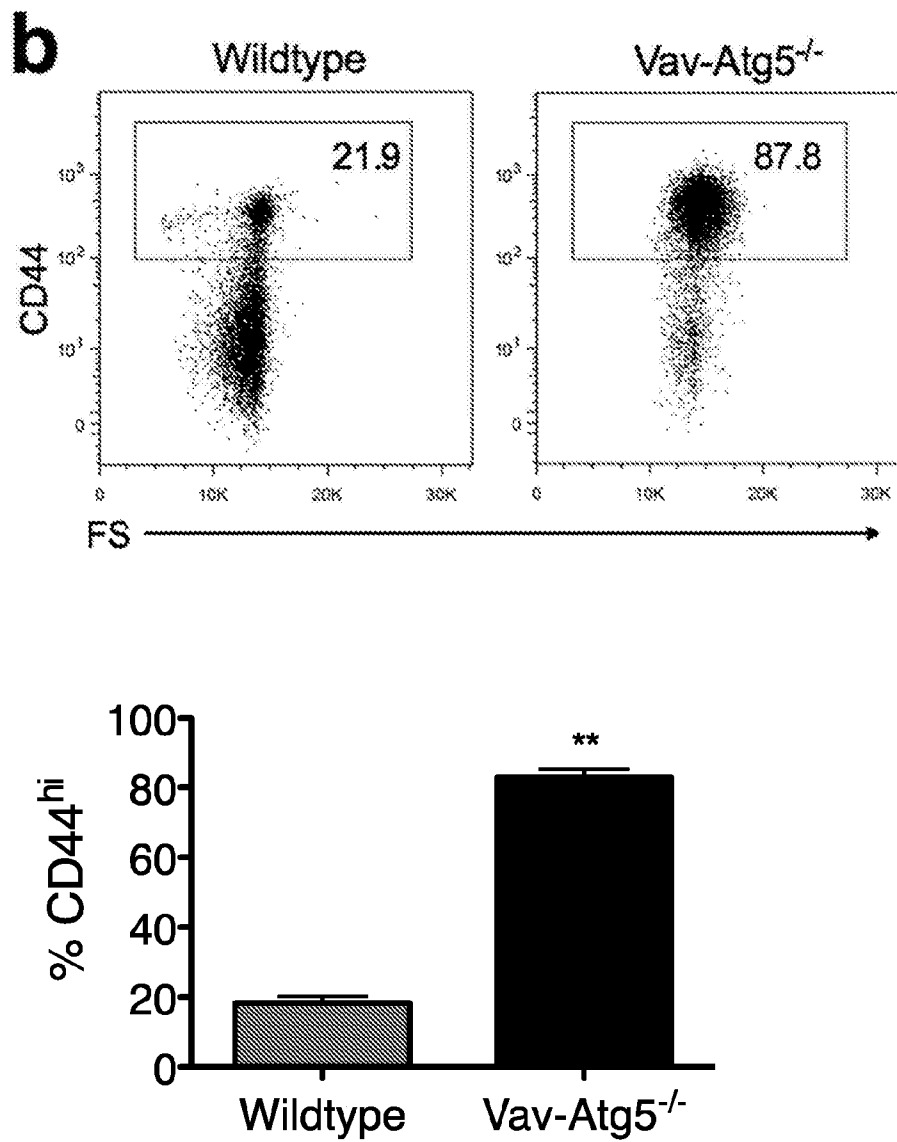


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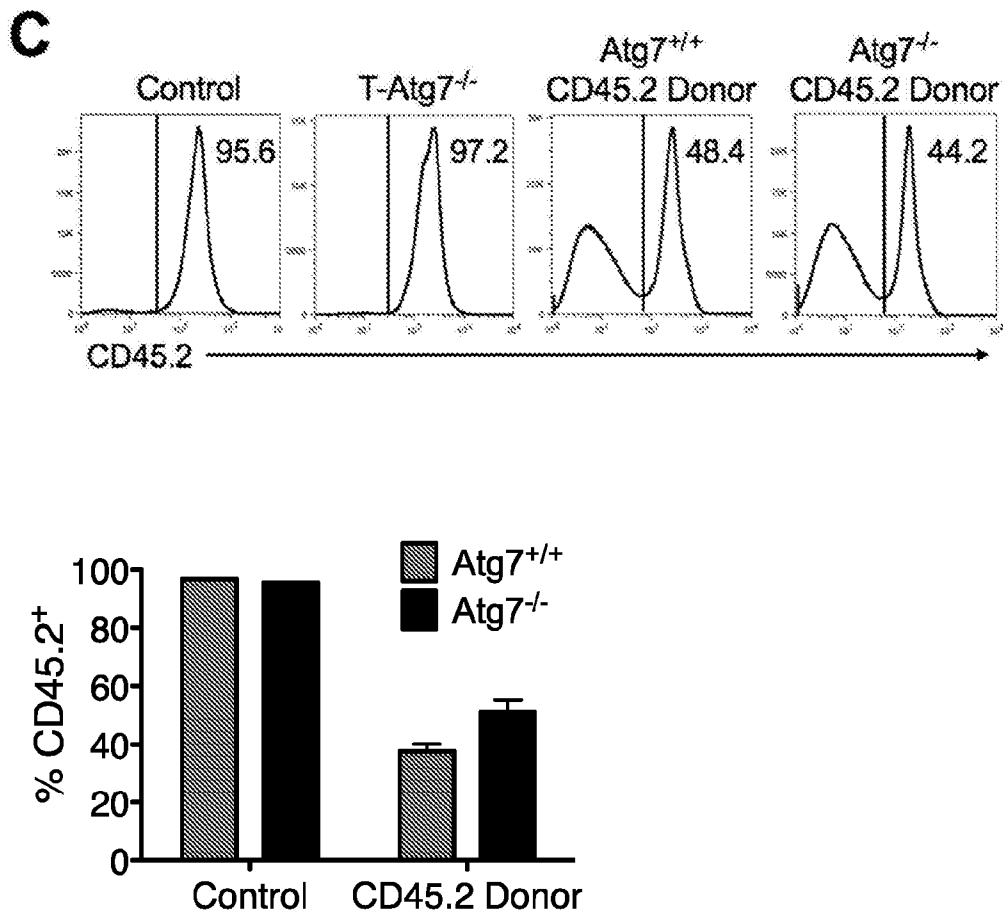


Figure 9

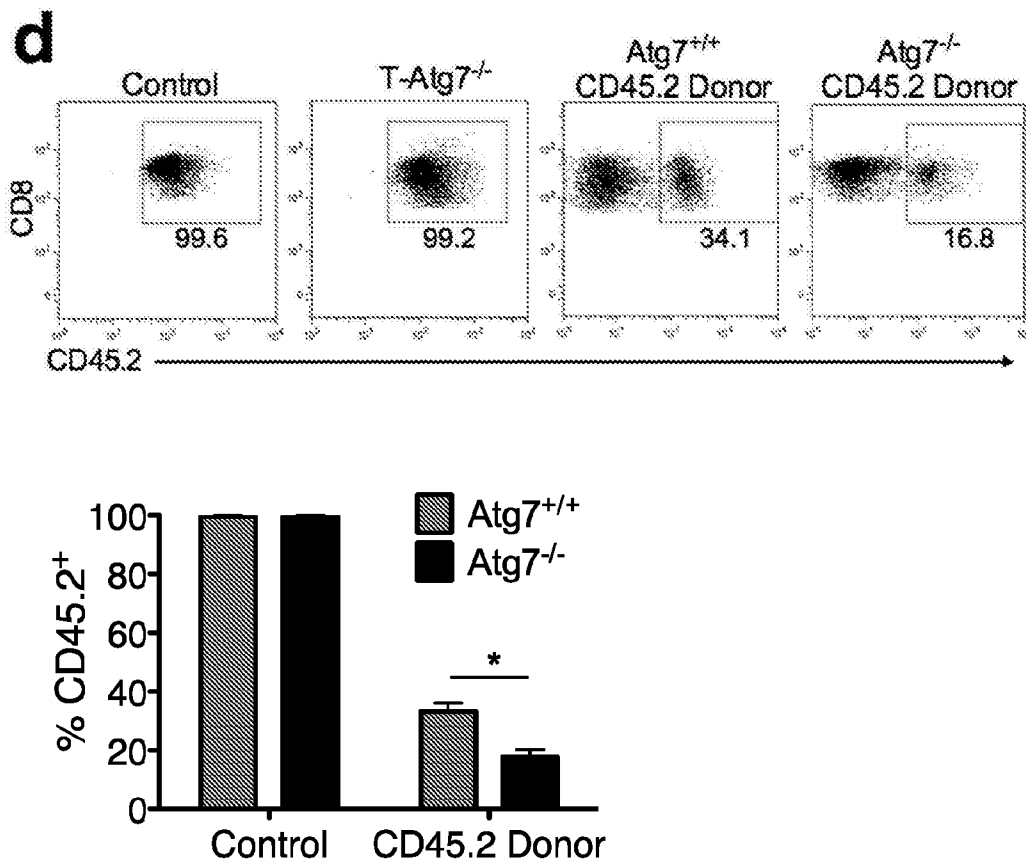


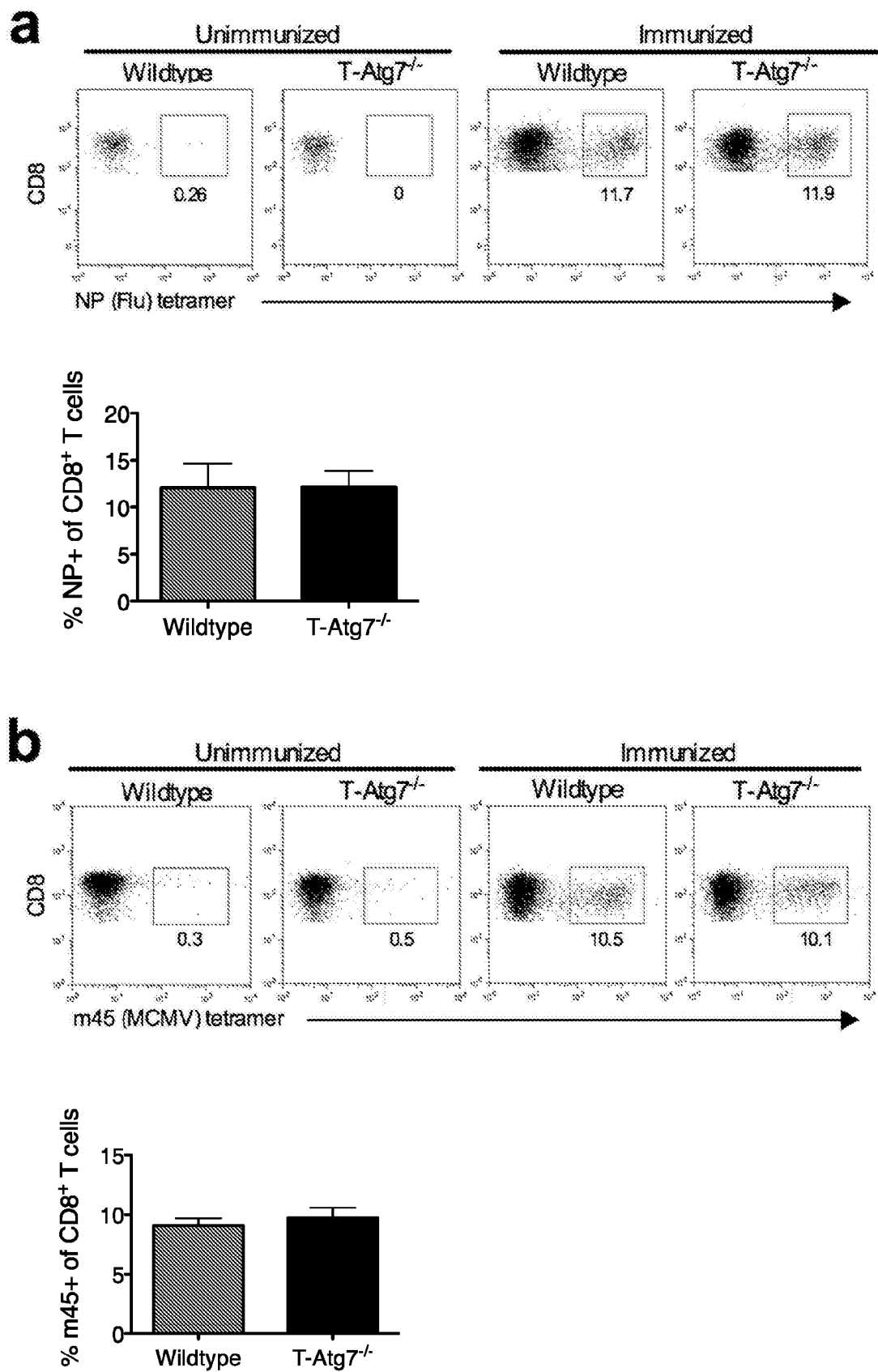
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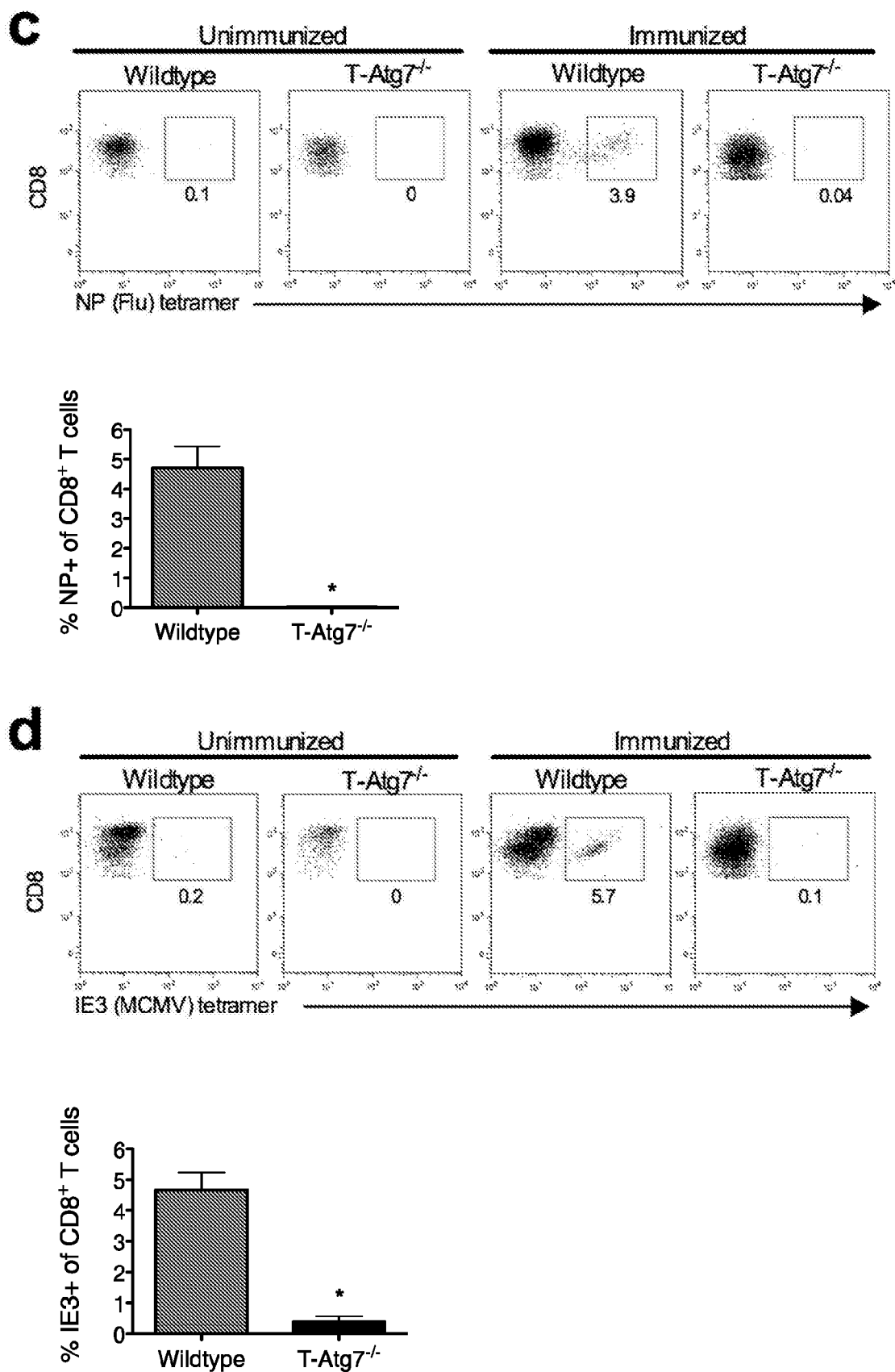
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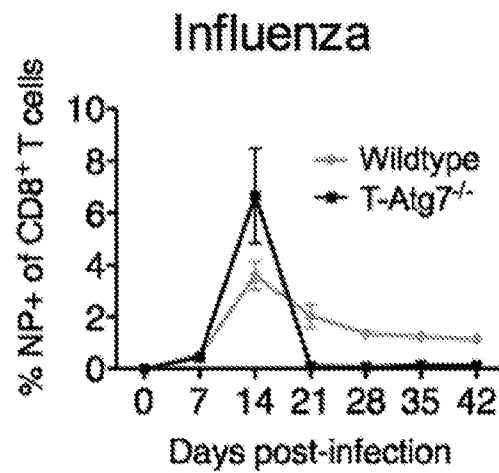
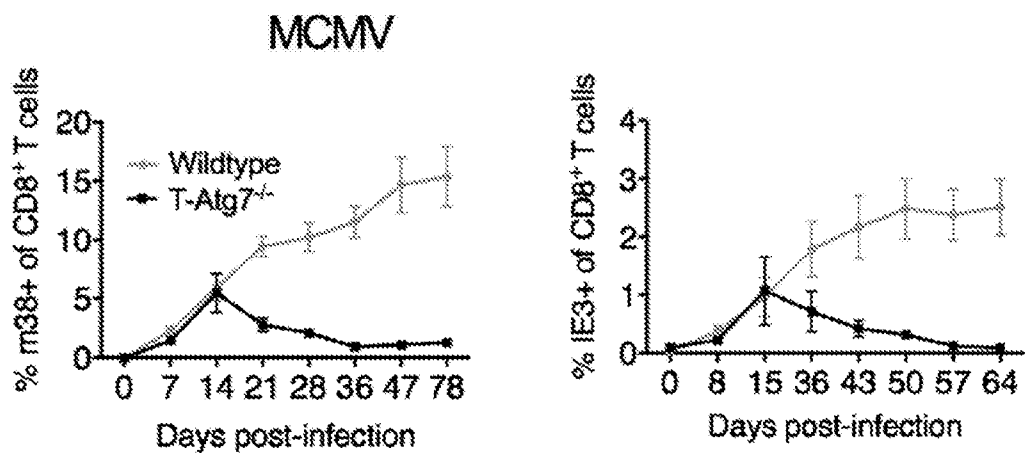
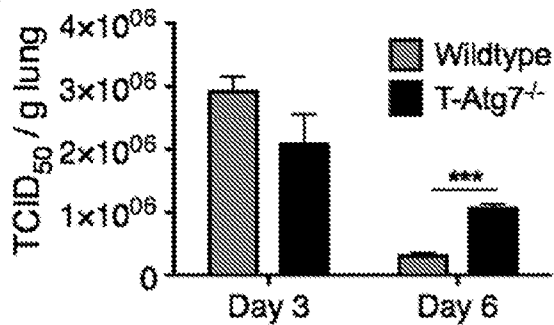
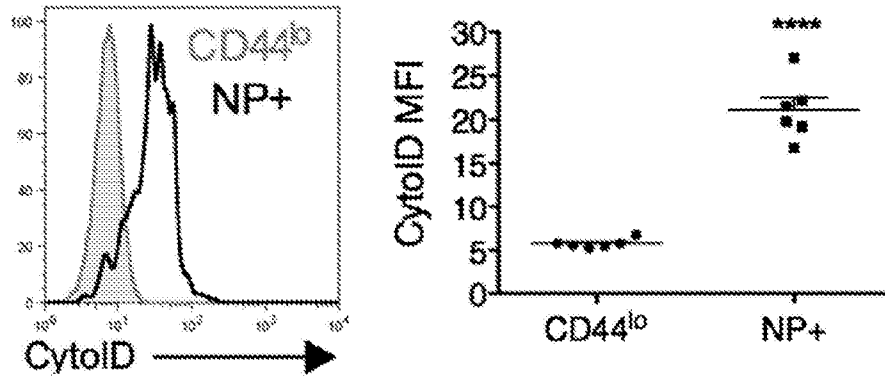
Figure 10**e****f****g**

Figure 10

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i

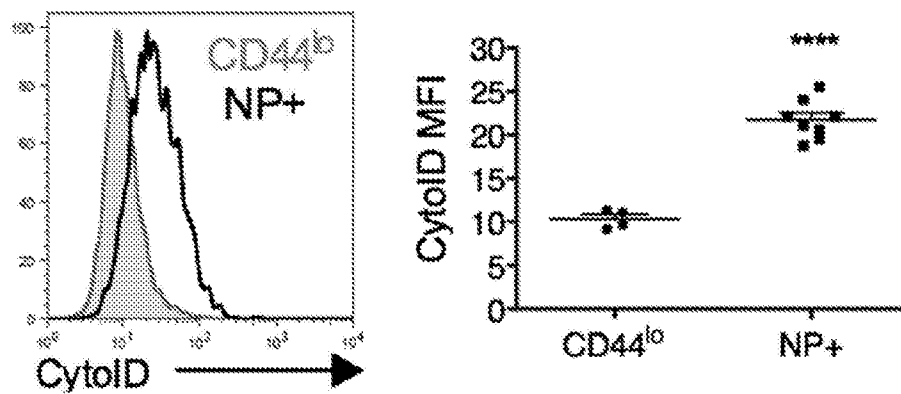


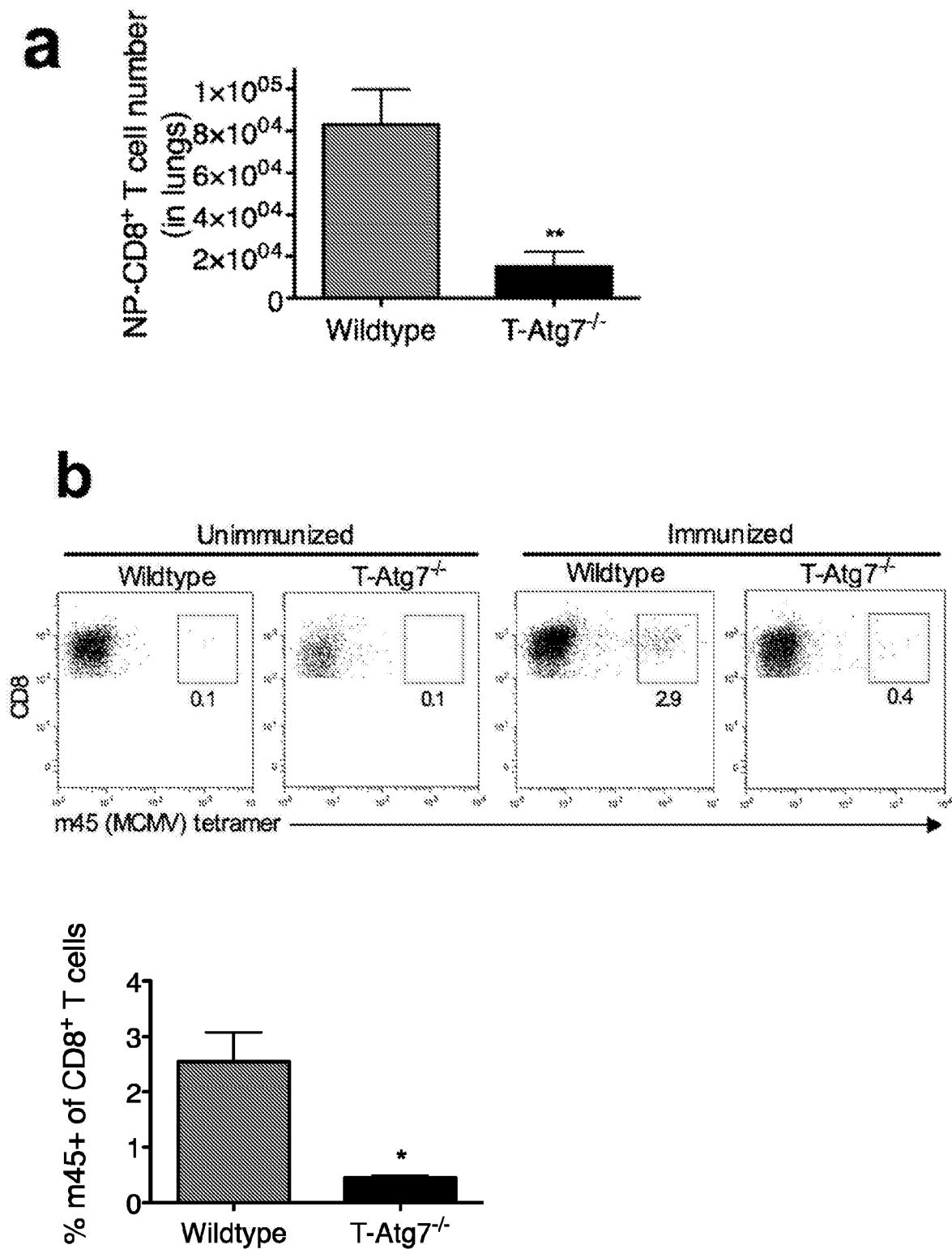
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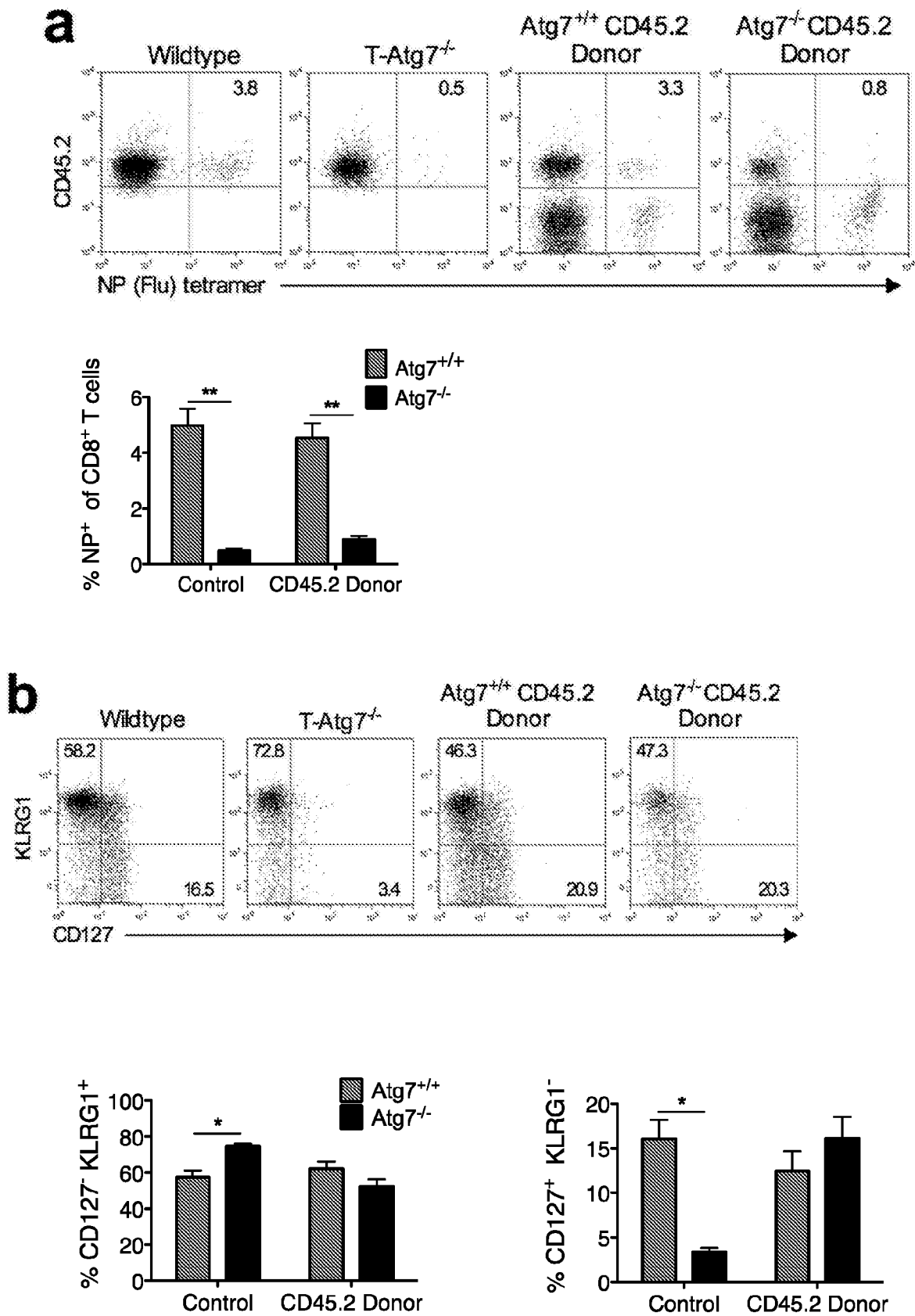
Figure 12

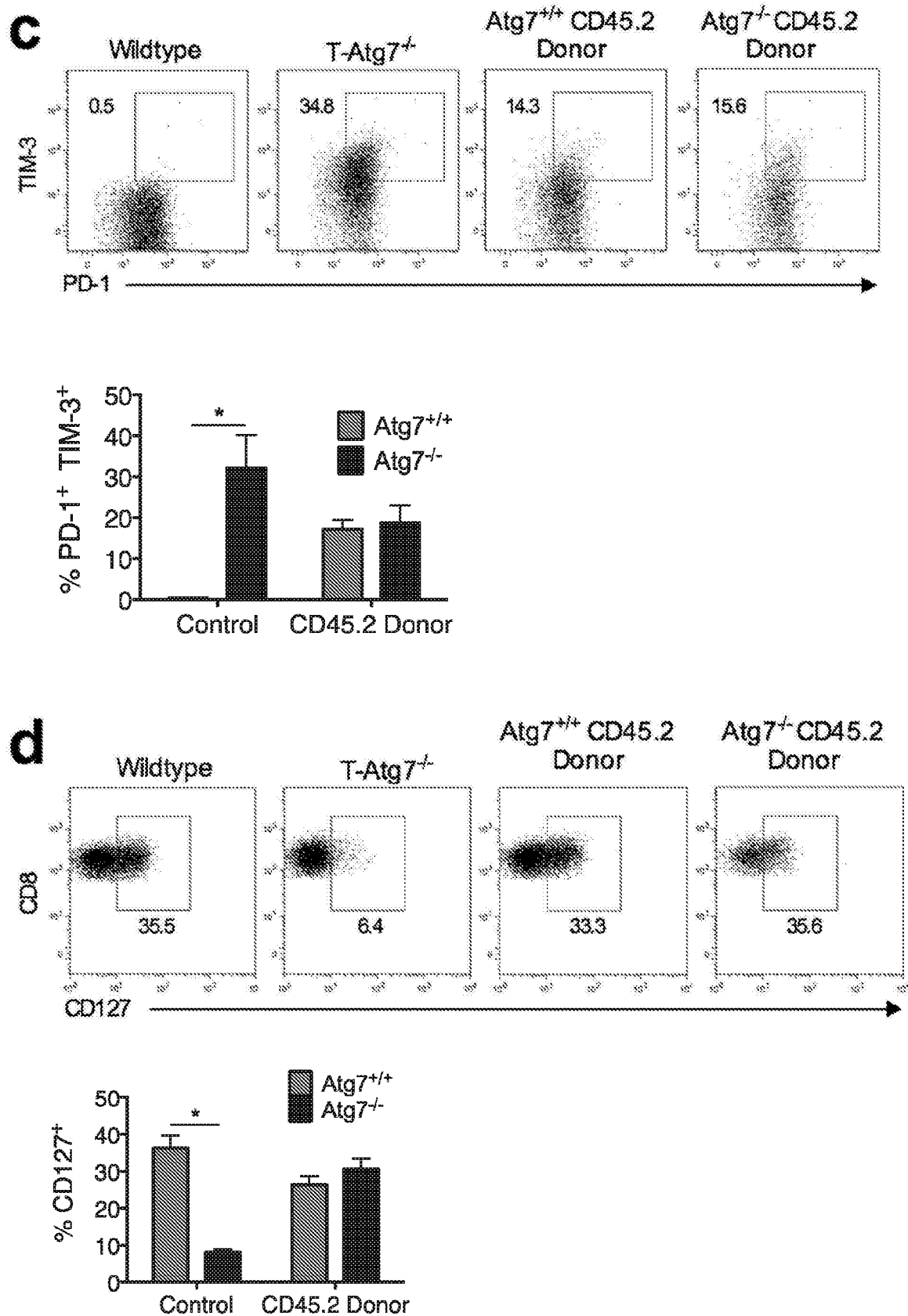
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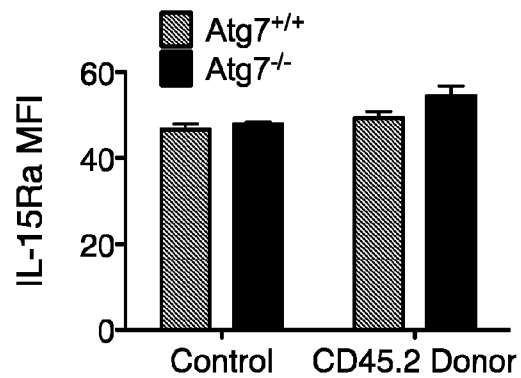
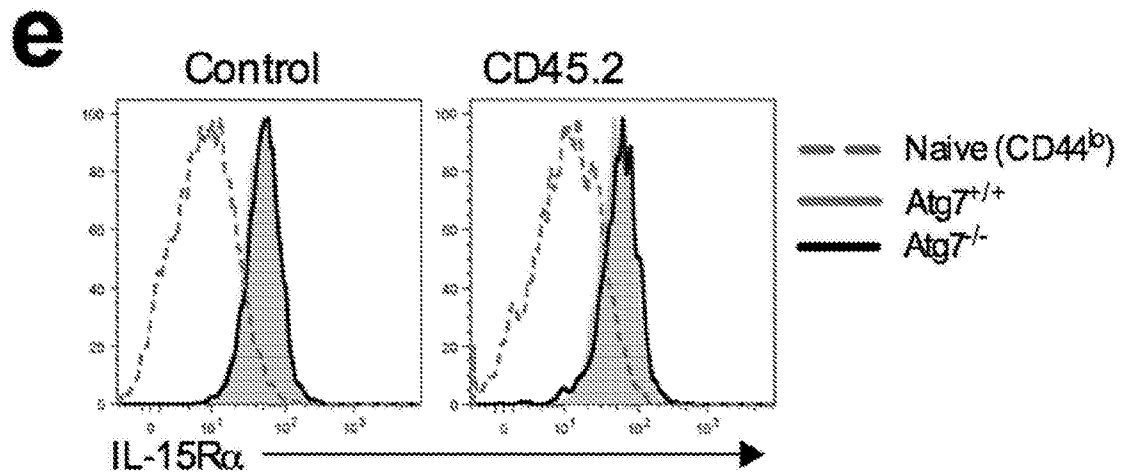
Figure 12

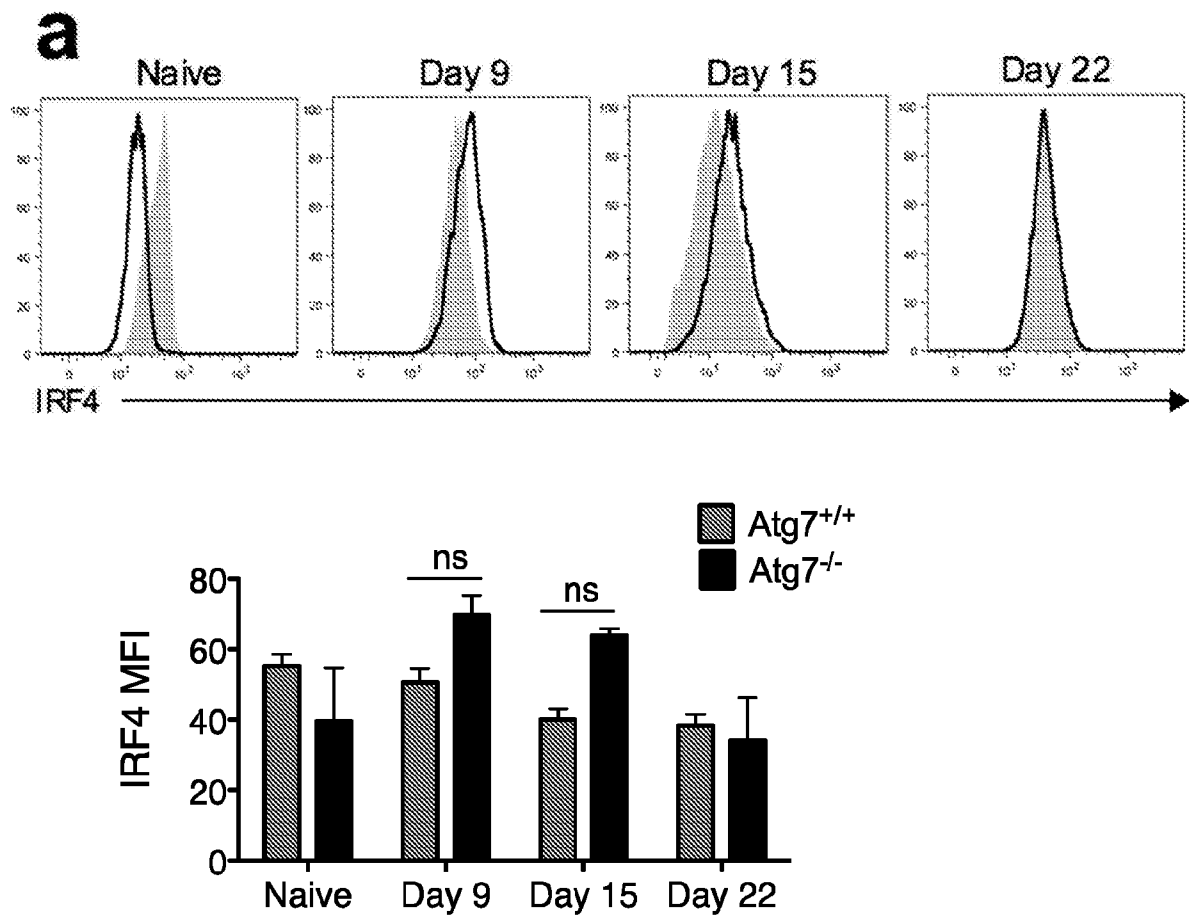
Figure 13

Figure 13

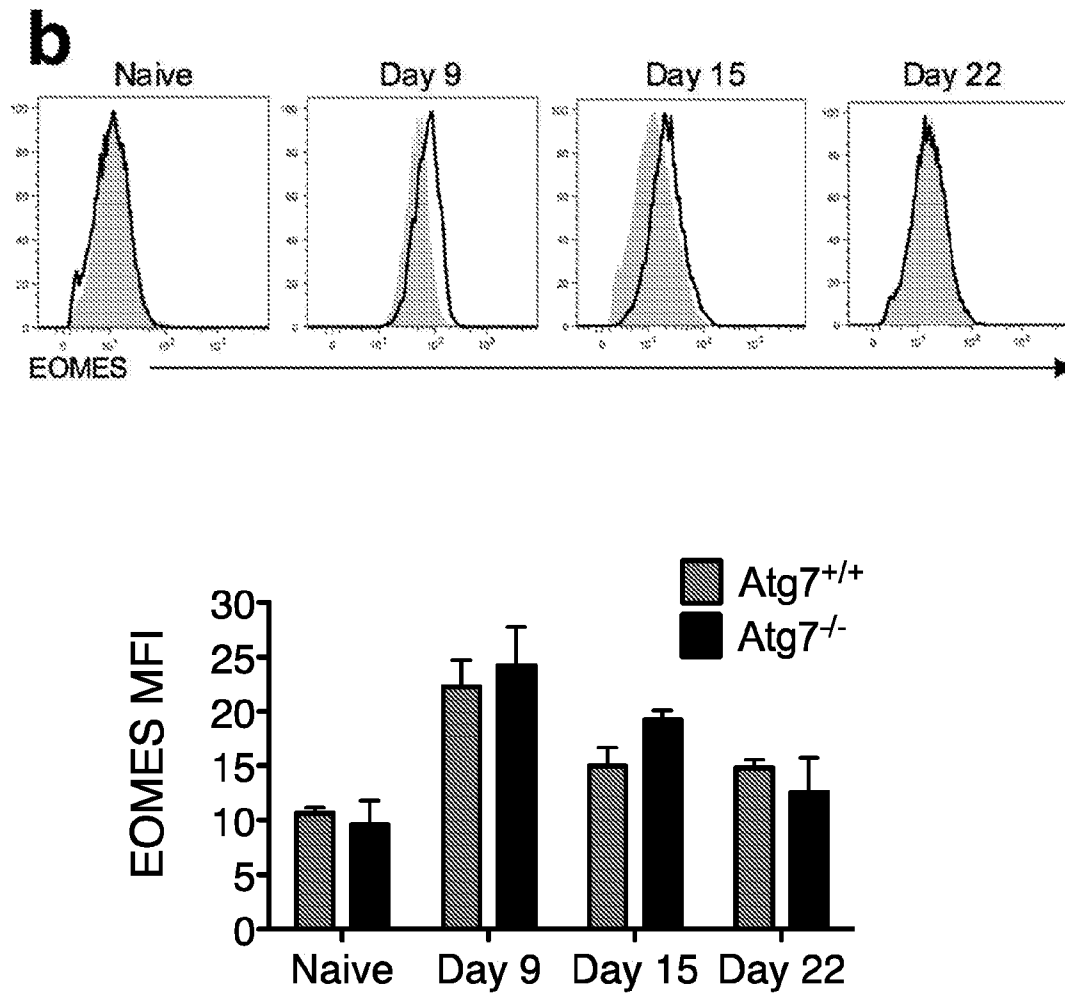


Figure 14

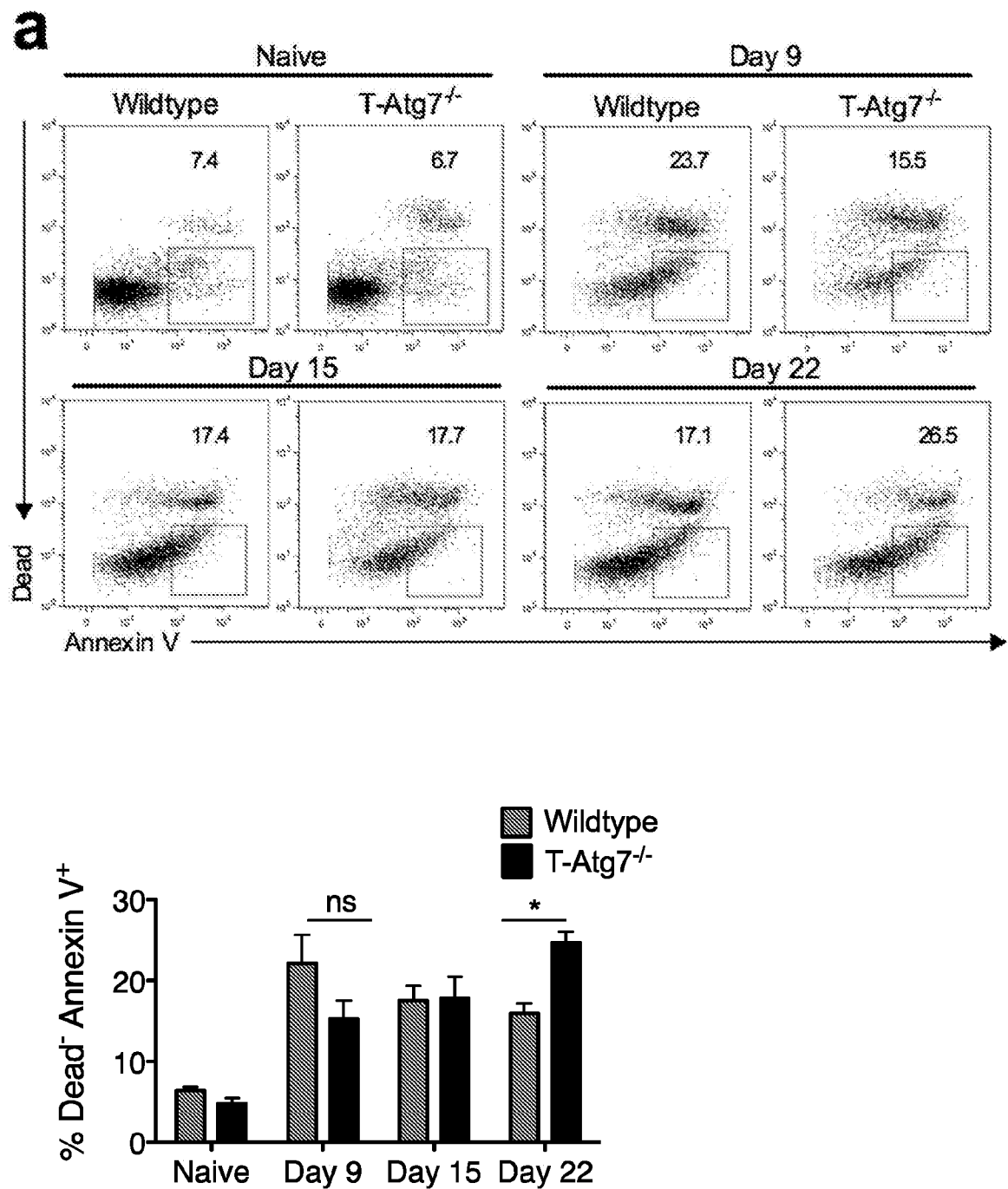


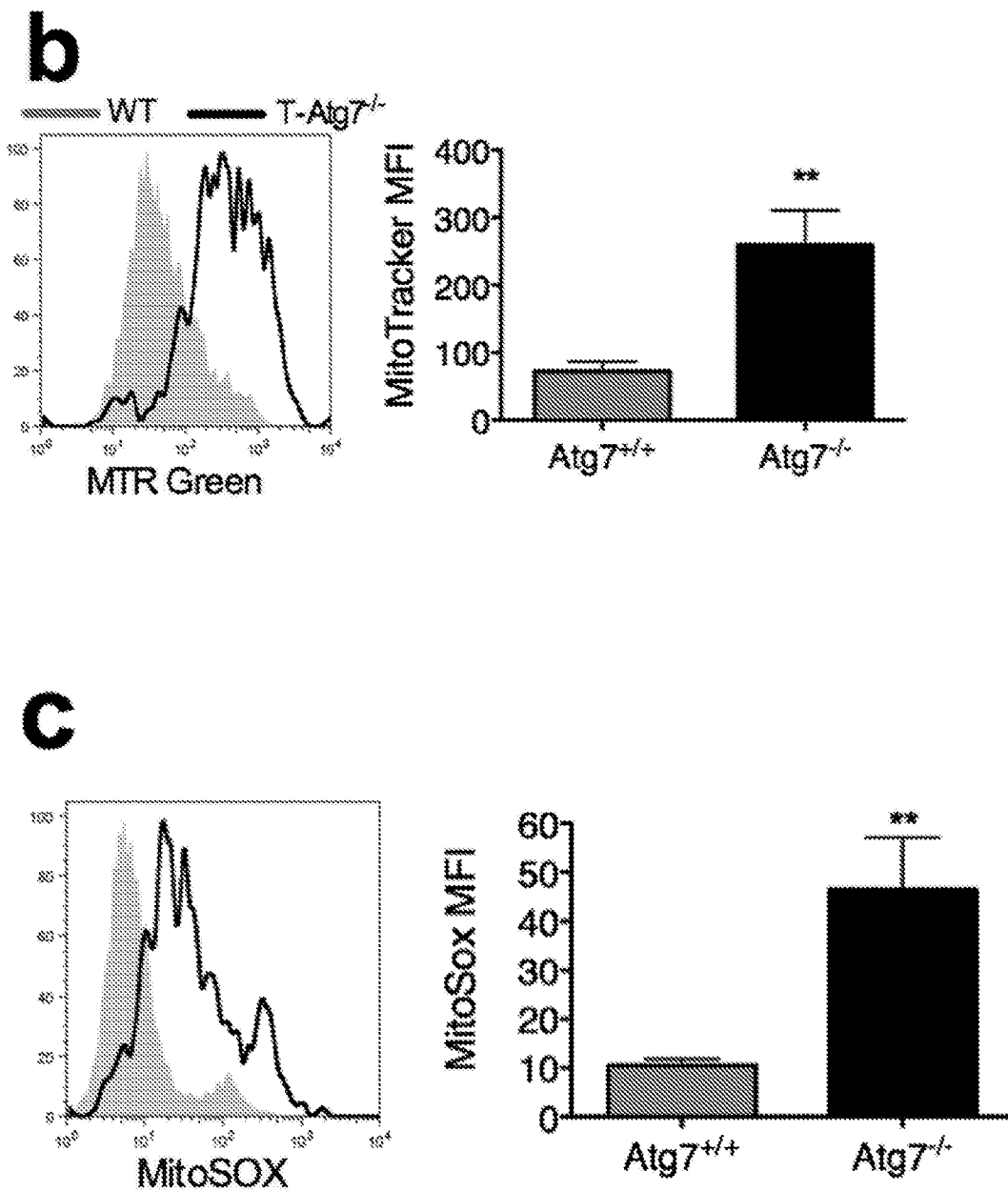
Figure 14

Figure 14

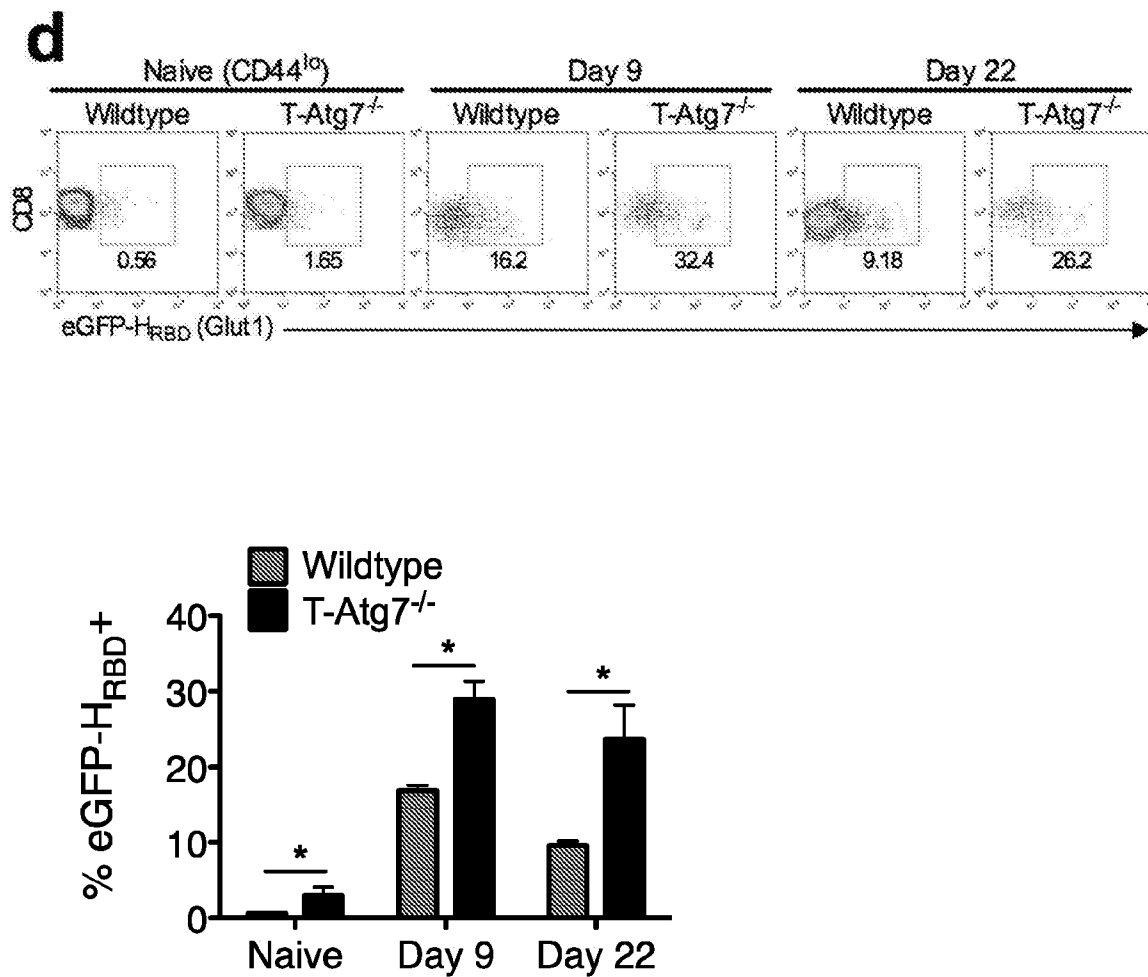


Figure 15

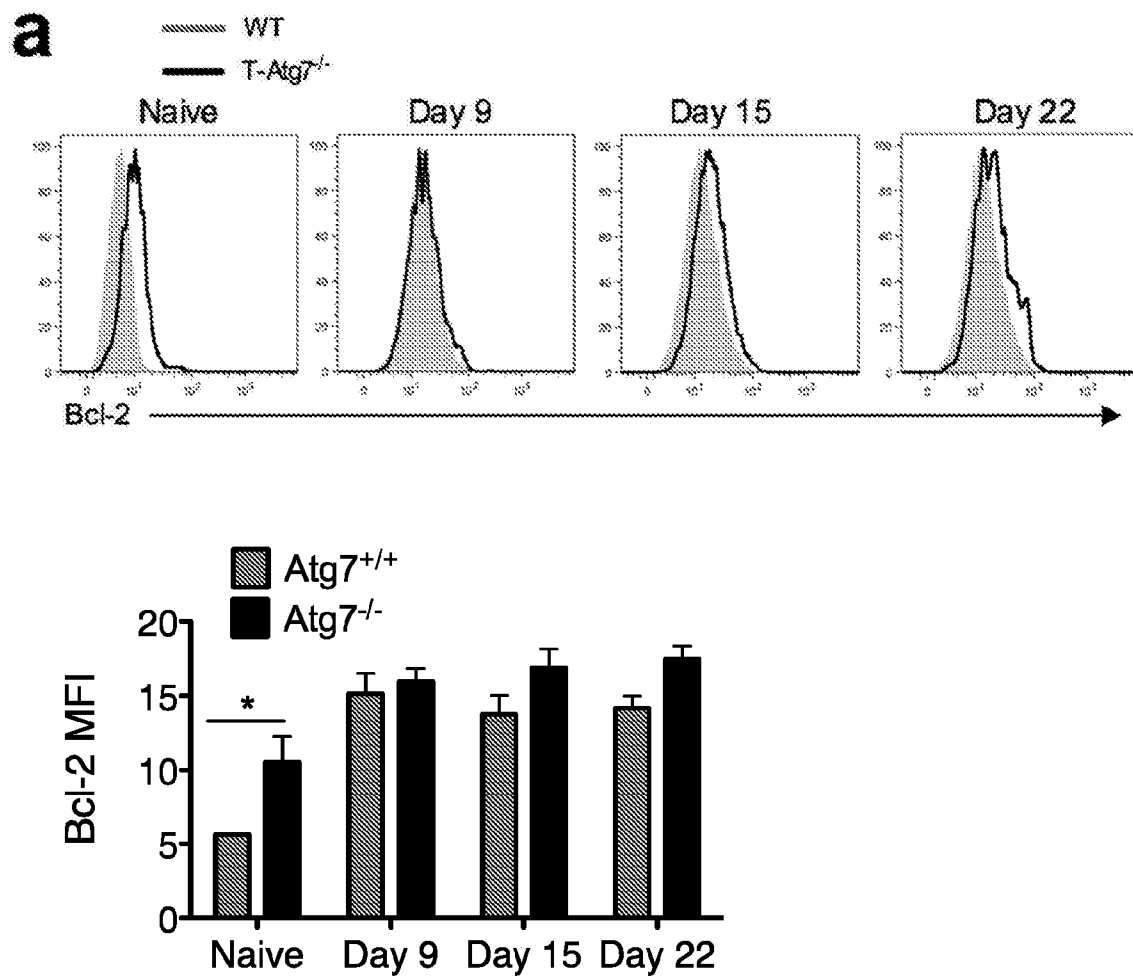


Figure 15

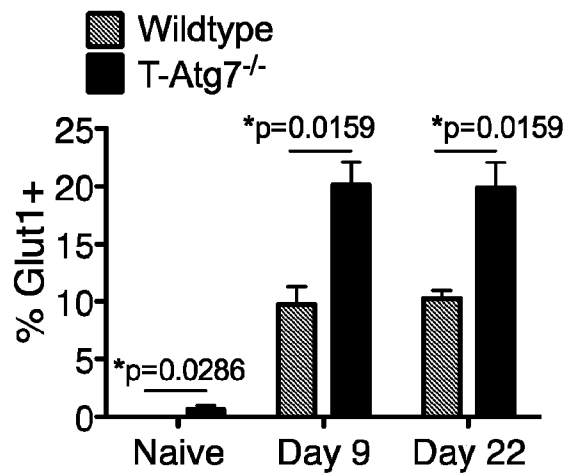
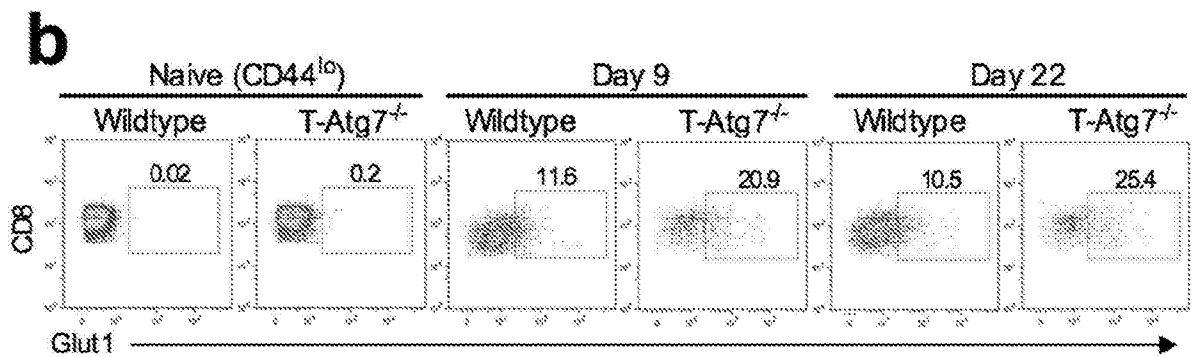


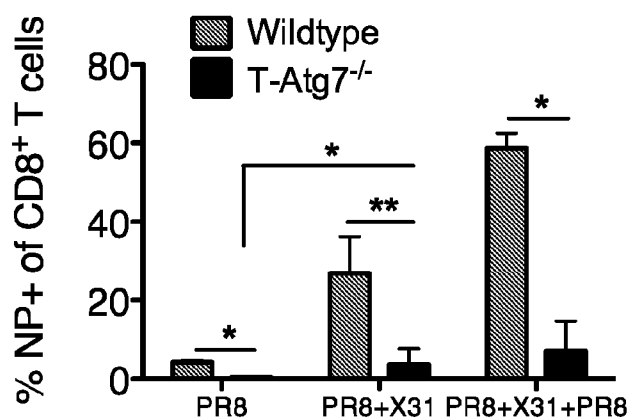
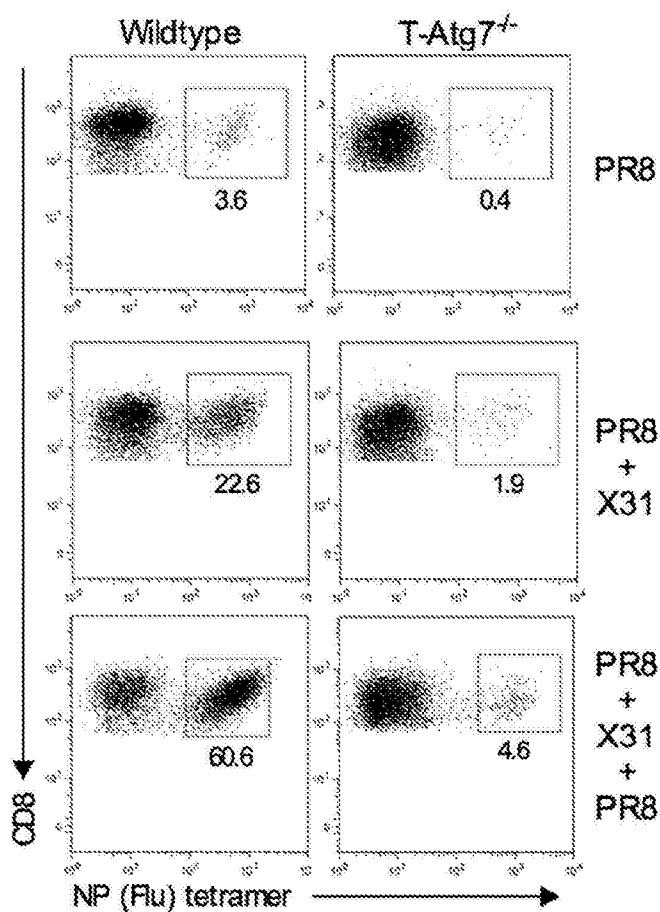
Figure 16**a**

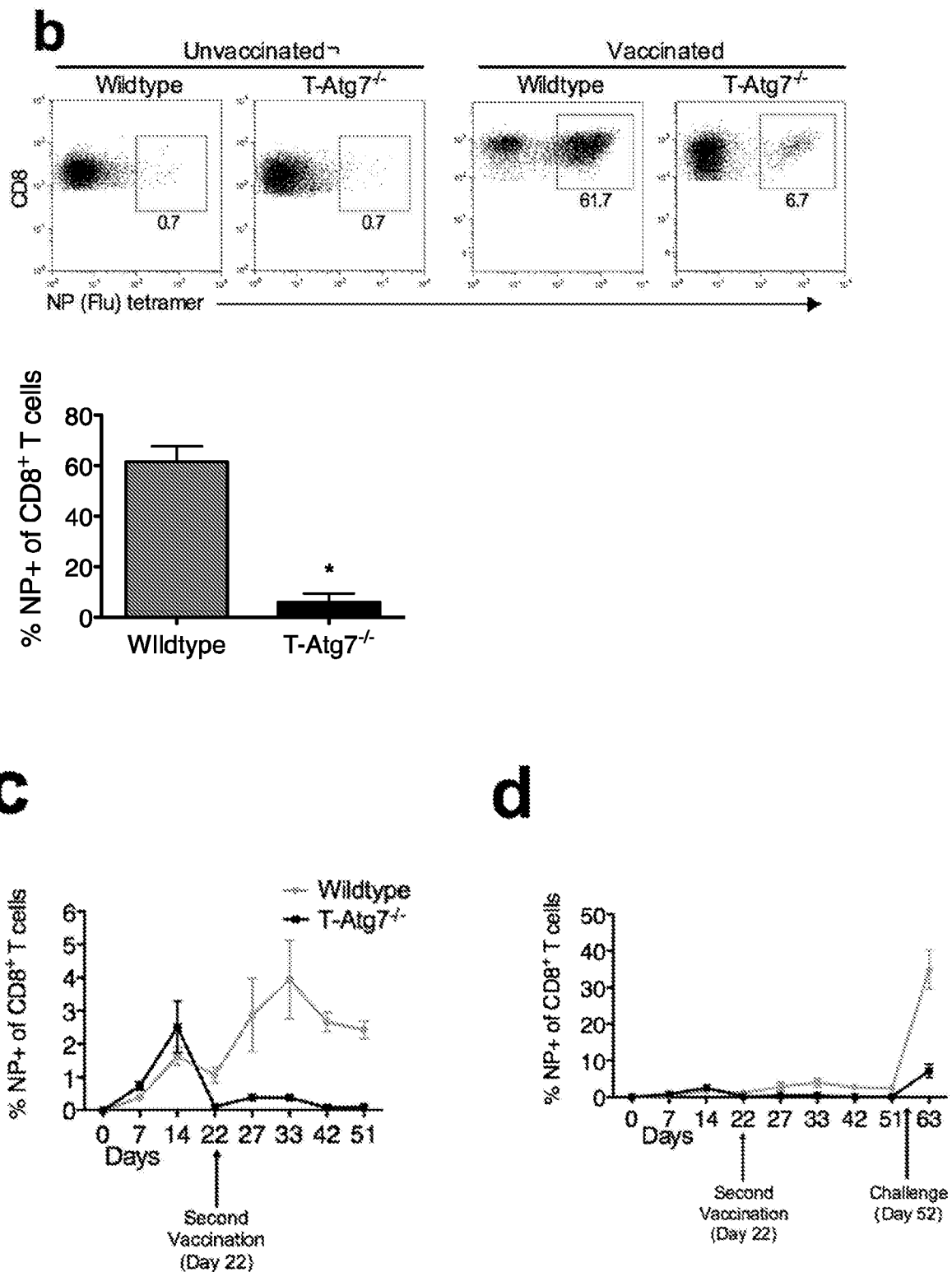
Figure 16

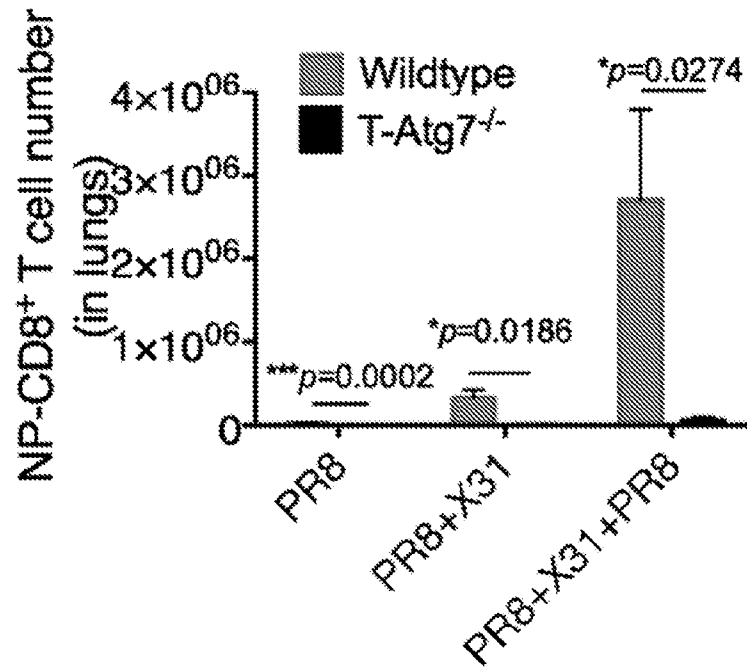
Figure 17**a**

Figure 18

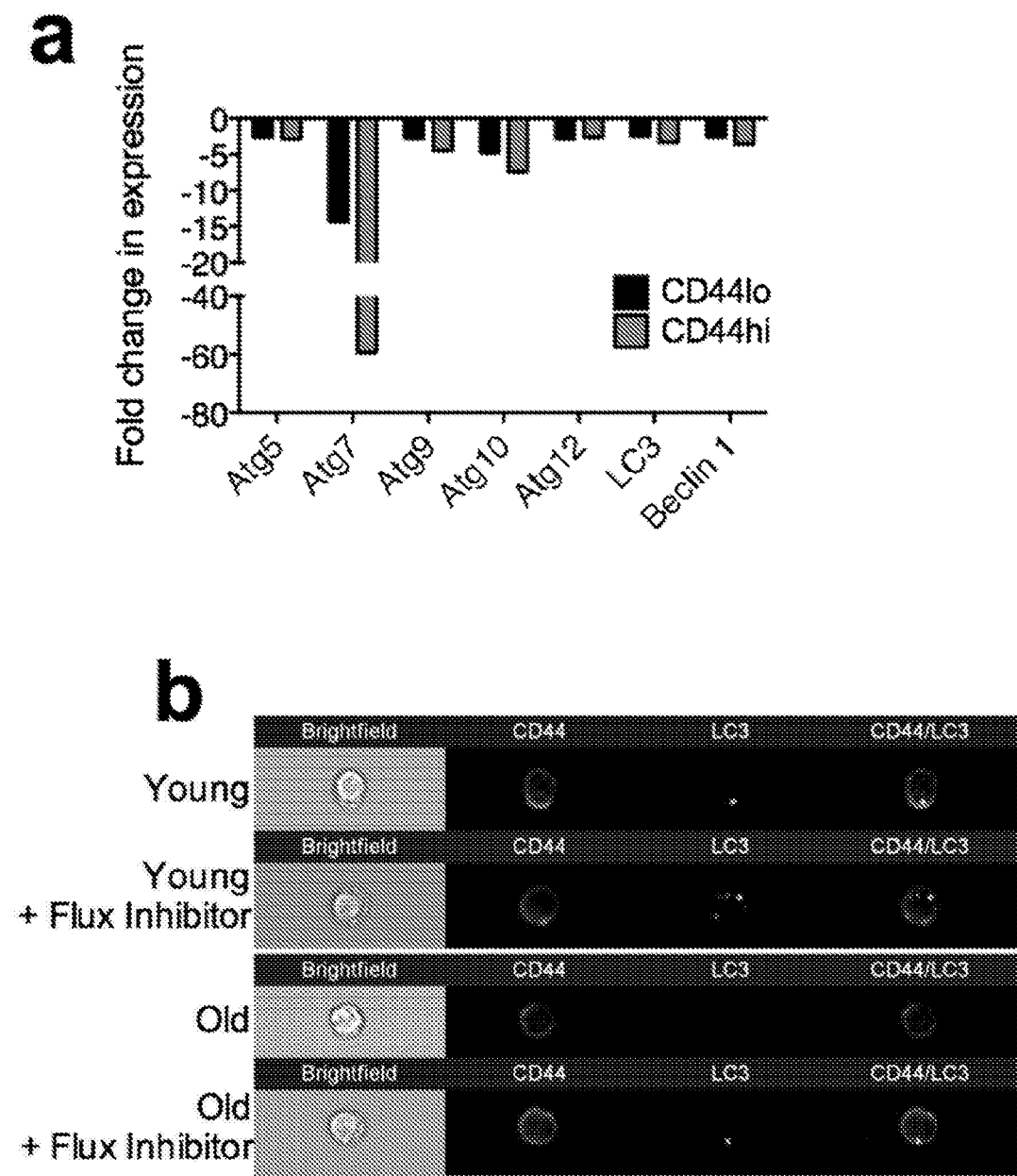
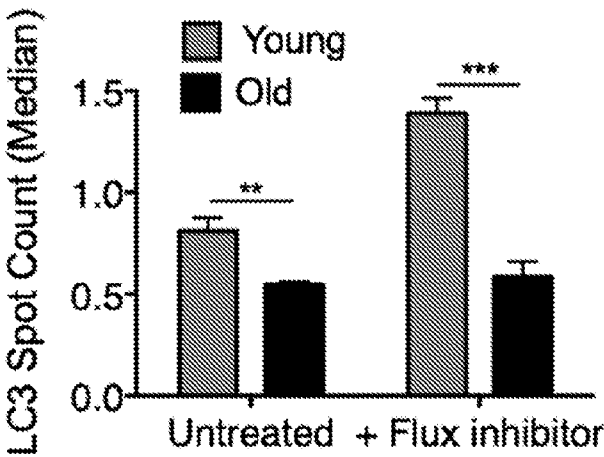


Figure 18

c



d

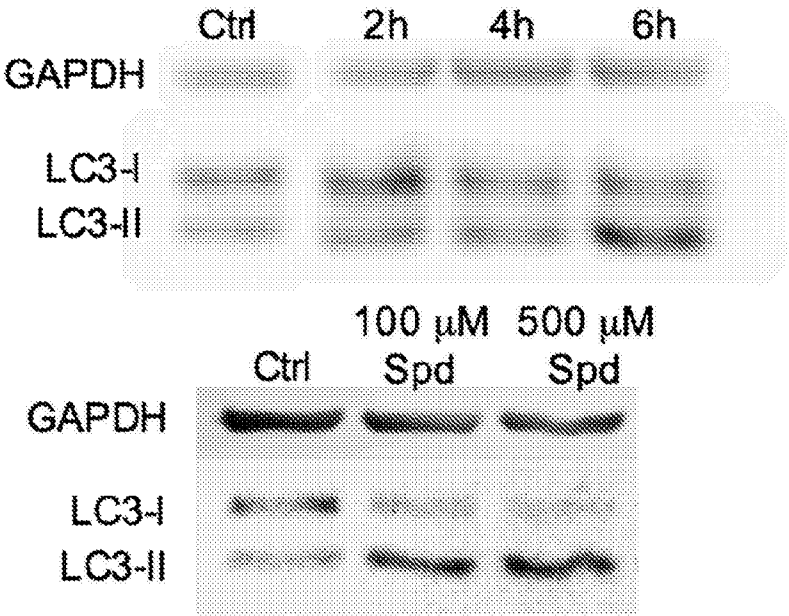


Figure 18

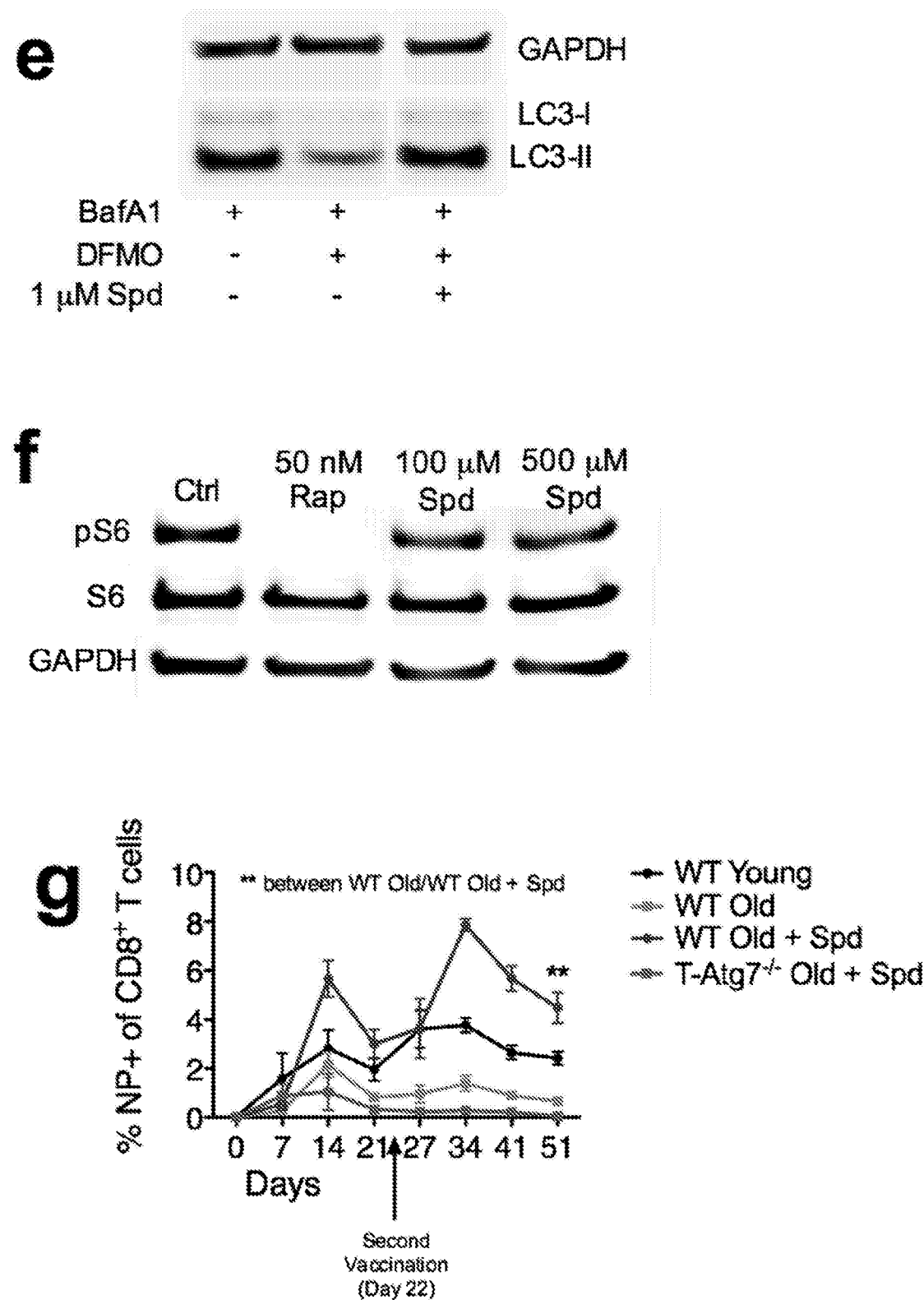


Figure 18

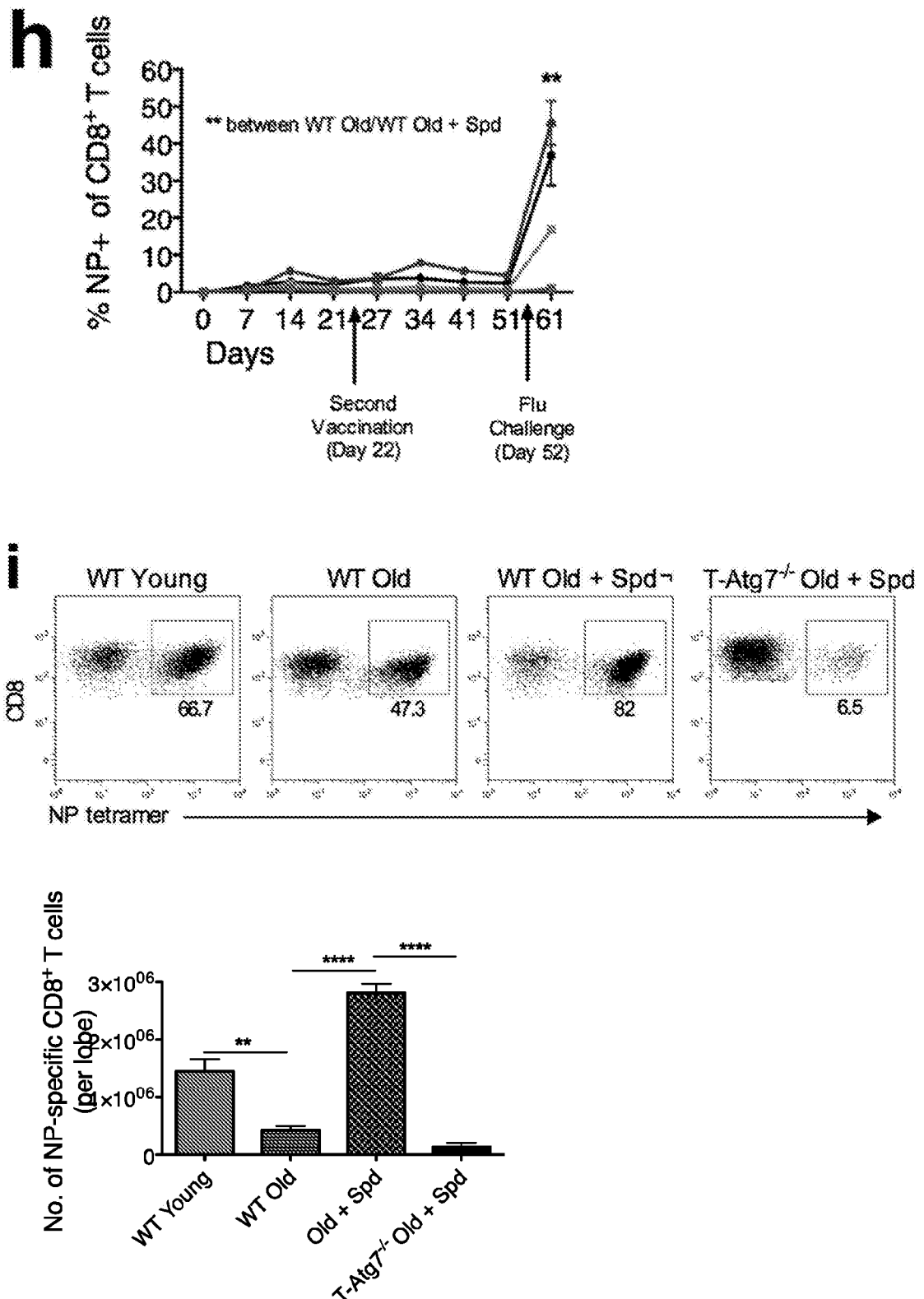
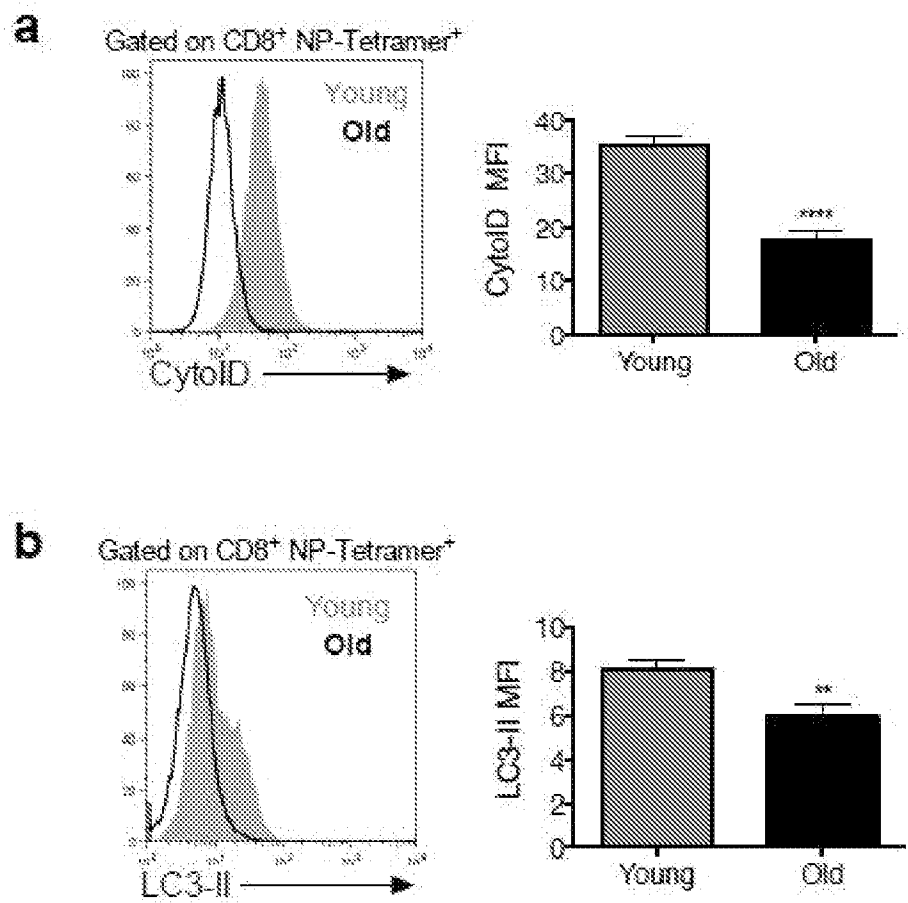


Figure 19



INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2015/050743

A. CLASSIFICATION OF SUBJECT MATTER INV. A61K31/132 A23L1/00 A61P43/00 A61K39/00 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) A61K A23L A61P 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 practicable, 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	WO 96/21356 A1 (UNIV VANDERBILT [US]) 18 July 1996 (1996-07-18) page 1, lines 10-19 page 4, line 31 - page 5, line 27 page 13, line 7 - page 14, line 14 claims 1-3 -----	1-4, 15-24, 26-37, 39,40
X	US 2006/171956 A1 (BAREHOLZ YECHZKEL [IL] ET AL) 3 August 2006 (2006-08-03) figures 1A-1D page 3, column 1, paragraph 39 page 5, paragraph 78-82 claims 30, 35 -----	1-4, 14-32 1-5, 14-33
Y	----- -/-	
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent 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 3 June 2015		Date of mailing of the international search report 15/06/2015
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 Collins, Sally

INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2015/050743

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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X	J.J. BALLEET ET AL: "Effect of polyamines on the proliferative response of T cells stimulated with antigen", EUROPEAN JOURNAL OF IMMUNOLOGY, vol. 5, 1975, pages 844-849, XP002740417, cited in the application	1,3,15, 21-24,32
Y	the whole document	16,17, 26-28
X	----- JOSEPH ET AL: "A new intranasal influenza vaccine based on a novel polycationic lipid-ceramide carbamoyl-spermine (CCS)", VACCINE, ELSEVIER LTD, GB, vol. 24, no. 18, 1 May 2006 (2006-05-01), pages 3990-4006, XP005421541, ISSN: 0264-410X, DOI: 10.1016/J.VACCINE.2005.12.017 cited in the application page 4000, column 2, paragraph 3.7 - page 4002, column 1, paragraph 3.9 page 4003, column 2, paragraph 3 - page 4004, column 2, paragraph 4 -----	1-14,17, 21,24, 25,38
X	US 2011/256161 A1 (BURNS MARK R [US] ET AL) 20 October 2011 (2011-10-20)	1
Y	page 1, paragraph 3 claims 1, 8-11 figure 3 -----	1-5, 14-33
A	WO 2011/008086 A1 (NUTRICIA NV [NL]; STAHL BERND [DE]; NAUTA ALMA JILDOU [NL]; GARSSSEN JO) 20 January 2011 (2011-01-20) the whole document -----	33-40
A	UNEMORI PATRICK ET AL: "T cell immunosenescence is associated with the presence of Kaposi's sarcoma in antiretroviral treated human immunodeficiency virus-infected persons", INFECTIOUS AGENTS AND CANCER, BIOMED CENTRAL LTD, LO, vol. 5, no. Suppl 1, 11 October 2010 (2010-10-11), page A74, XP021081948, ISSN: 1750-9378, DOI: 10.1186/1750-9378-5-S1-A74 the whole document ----- -/--	6,33

INTERNATIONAL SEARCH REPORT

International application No
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JANET E MCELHANEY ET AL: "The unmet need in the elderly: How immunosenescence, CMV infection, co-morbidities and frailty are a challenge for the development of more effective influenza vaccines", VACCINE, ELSEVIER LTD, GB, vol. 30, no. 12, 5 January 2012 (2012-01-05), pages 2060-2067, XP028460646, ISSN: 0264-410X, DOI: 10.1016/J.VACCINE.2012.01.015 [retrieved on 2012-01-20] the whole document -----	6,33

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International application No

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