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(71) Applicant: **THE JOHNS HOPKINS UNIVERSITY**
[US/US]; 3400 N. Charles Street, Baltimore, Maryland
21218 (US).

(72) Inventors: **SCHNECK, Jonathan**; 5 Yeatman Court, Silver
Spring, Maryland 20902 (US). **CHIU, Yen-Ling**; c/o
THE JOHNS HOPKINS UNIVERSITY, 3400 N. Charles
Street, Baltimore, Maryland 21218 (US).

(74) Agent: **HEMMENDINGER, Lisa M.**; Banner & Witcoff,
Ltd., 1100 13th Street NW, Suite 1200, Washington, Dis-
trict of Columbia 20005 (US).

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(54) Title: METHODS OF INDUCING T CELL POLYFUNCTIONALITY

(57) Abstract: The ability of individual T cells to perform multiple effector functions is crucial for protective immunity against vir-
uses and cancer. This polyfunctionality is frequently lost during chronic infections. This disclosure provides methods of inducing T
cell polyfunctionality to treat chronic viral infections and cancer



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METHODS OF INDUCING T CELL POLYFUNCTIONALITY

[1] Each reference cited in this disclosure is incorporated herein by reference in its entirety.

[2] This invention was made with government support under NIH grants NCI RO1 CA 108835, NIAID PO1 AI072677 AI 080313, and AI 36219. The government has certain rights in the invention.

FIELD OF THE INVENTION

[3] The invention is in the field of immunology.

BACKGROUND

[4] A growing consensus indicates that T cells capable of simultaneously producing multiple effector functions, referred to as “polyfunctional” T cells, are a key subset in the development of effective immune responses against pathogens and cancer (1–7). These T cells, in addition to producing cytokines such as IL-2, TNF- α , and IFN- γ , produce chemokines and also display cytolytic function. In contrast to acute infection, optimal polyfunctional memory T cell responses are lost in chronic infections and cancer, both in humans and mice (5, 8, 9). These cells have been characterized as “exhausted” (8, 10) and factors promoting the development of T cell exhaustion include persistent and high levels of antigen stimulation (11, 12), DC inhibition (3), and upregulation of inhibitory receptors, such as PD-1, on T cells (13–17). While recent work has highlighted a role for PD-1 and other inhibitory receptors in T cell exhaustion, blockade of inhibitory receptor signaling in HIV-specific T cells had only a moderate effect in reversing the exhaustion phenotype and increasing T cell polyfunctionality (14, 15), thus indicating that additional molecular mechanisms are involved in the inhibition of T cell polyfunctionality.

[5] Differences in T cell polyfunctionality correlate with differences in memory T cell formation in response to immunization as well as to viral infection. Regulation of vaccine-induced adaptive immune responses is complex and, in part, dependent on antigen dose (1). In animal models, high-dose vaccination results in inferior T cell polyfunctionality, poor memory formation and weaker immune protection as compared with optimal dose vaccination (4, 18). As a result, polyfunctional T cells are more than just a “marker” of

protective immune response. The molecular mechanisms linking high-dose antigenic stimulation with inferior polyfunctionality and poor memory formation remain largely unclear.

[6] Collectively, these data indicate the need to understand the molecular control of T cell polyfunctionality, especially in the context of antigen dose. Elucidating the molecular details underlying T cell polyfunctionality could provide additional insights into T cell exhaustion and serve as a basis for vaccine design by optimizing T cell activity against virus infection or cancer.

[7] Polyfunctionality is crucial for protective immunity against viruses and cancer (Seder et al., 2008; Wherry, 2011). It is widely accepted that a robust T cell immune response is not only dependent on the absolute cell number, but is also critically dependent on cell quality. Polyfunctional T cells, characterized by the ability to execute multiple effector functions, are a hallmark of healthy, non-exhausted memory T cell response against acute infection, such as influenza infection (Joshi and Kaech, 2008; Weng et al., 2012; Wherry, 2011; Wherry and Ahmed, 2004). On the other hand, during chronic infectious diseases such as HIV and HCV, dysfunctional antigen-specific T cells are frequently formed due to repetitive stimulation and are associated with worse disease outcomes (Betts et al., 2006; Ciuffreda et al., 2008; Frebel et al., 2010; Rehr et al., 2008). Besides infectious diseases, loss of polyfunctional T cell responses is also reported in human tumors (Baitsch et al., 2011; Tran et al.) and successful anti-tumor immunotherapy treatments correlate with the induction of polyfunctional T cells (Ding et al., 2012; Yee, 2010; Yuan et al., 2008).

BRIEF DESCRIPTION OF THE FIGURES

Figures for Examples 1-8

[8] **FIGS. 1A-D.** Results of experiments demonstrating that activation of Wnt signaling reprograms memory T cells to a polyfunctional state.

[9] **FIGS 2A-F.** Results of experiments demonstrating that Wnt pathway activation with TWS119 induces polyfunctional influenza-specific T cells with a central memory phenotype. Human CD8⁺ T cells were stimulated with autologous, M1-peptide loaded moDCs for two weeks (stimulations were given on D0 and D7). The culture was also treated with different amount of TWS119, as indicated. Tetramer staining and cell counts were determined on D7 and D14. **FIG. 2C and FIG. 2D,** D14 CD8⁺ T Cells induced with different concentration of TWS119 were harvested and re-challenged with target T2 cells loaded with M1 peptide for

six hours and analyzed for their effector functions. **FIG. 2C**, Representative intracellular cytokine staining for polyfunctionality analysis. Top row: Intracellular cytokine staining for IL-2 versus CD107. Numbers in the right upper quadrant represent the percentage of IL-2 and CD107a double-positive cell population. Bottom row: Intracellular cytokine staining for TNF α versus MIP-1 β . Numbers in the right upper quadrant represent the percentage of TNF α and MIP-1 β double-positive cell population. **FIG. 2D**, Polyfunctionality pie charts. Each slice of pie represents the percentage of cells expressing between 1~5 effector functions. To compare between pie charts, permutation test with 10,000 repetitions was used using SPICE. **FIG. 2E**, TWS119 treatment significantly increased the absolute number of 5+ polyfunctional cells during antigen-specific expansion. Numbers were calculated based on a starting CD8+ culture composed of 100,000 cells. **FIG. 2F**, D14 CD8+M1+ Cells were also analyzed for their surface marker expression. TWS119 3mM, blue; 1.5mM, green; 0.7mM, orange; 0mM, red. Histograms were gated on CD8+tetramer+ cells. *: P value<0.05. **: P value<0.01. The results shown are from five different donors.

[10] **FIGS. 3A-E**. Results of experiments demonstrating that TWS119 treatment enhances polyfunctionality in terminally differentiated CMV-specific T cells. **FIG. 3A**, Ex vivo CMV-pp65-specific response. **FIG. 3B**, Representative tetramer staining of Human CD8+ T cells stimulated with CMV-pp65-NLV loaded moDCs for two weeks (D14). TWS119 were added in culture during stimulation at the indicated concentration. **FIG. 3C and FIG. 3D**, D14 cells were rechallenged with pp65-loaded T2 target cells for six hours to assess their polyfunctional response. Total numbers of pp65-specific 5+ polyfunctional cells were calculated as in **FIG. 2**. Similar to what has been observed with M1-specific response, there is enhanced polyfunctional response in TWS119 treated cells. **FIG. 3E**, TWS119 treated cells also express higher level of CD62L and CD28. TWS119 3mM, blue; 1.5mM, green; 0.7mM, orange; 0mM, red. Histograms were gated on CD8+CMV-tetramer+ cells. *: P value<0.05. **: P value<0.01.

[11] **FIGS. 4A-D**. Results of experiments demonstrating that the effects TWS119 on CD62L expression and polyfunctionality are seen in divided cells and are independent of dendritic cells. Human CD8+ T cells were stimulated with M1 or CMV-peptide loaded moDCs for two weeks in the presence or absence of TWS119. Before the second moDC stimulation, T cells were labeled with CFSE to monitor population divisions. On D14, cells

were either harvested and stained with CD8, tetramer and CD62L, or rechallenged with M1-labeled T2 cells for six hours for intracellular cytokine staining. **FIG. 4A**, Representative staining showing CD62L by CFSE dilution among CD8⁺ M1-tetramer⁺ cells. TWS119 decreased the proliferation of tetramer⁺ cells, but almost all tetramer⁺ cells divided. **FIG. 4B**, Representative result of cytokine production by CFSE dilution among CD8⁺ M1-tetramer⁺ cells. T cells induced with TWS119 exhibited higher level of IL-2 or TNF α in divided cells. **FIG. 4C and FIG. 4D**, The effect of Wnt pathway activation was observed when T cells were expanded with aAPCs. Purified CD8⁺ T cells were stimulated with M1-loaded or pp65-loaded aAPCs weekly for two weeks. Tetramer specificity (**FIG. 4C**) and T cell polyfunctionality (**FIG. 4D**) were determined weekly. *: P value<0.05.

[12] **FIGS. 5A-E**. Results of experiments demonstrating that Wnt pathway activation induced a long-standing polyfunctional phenotype associated with other stemness features. **FIG. 5A**, M1-tetramer positive T cells induced by moDCs in the presence (Blue) or absence of TWS119 (Red) were studied for their anti-apoptotic protein expression levels on D14. Numbers shown indicate the MFI value for each staining. **FIGS. 5B-5E**, D14 M1-specific cells were further cultured in the presence of IL-15 (25ng/ml) for 7 days. CFSE dilution, phenotype and polyfunctionality were analyzed on D21. **FIG. 5B**, Representative CFSE dilution of M1-tetramer specific cells measured by flow cytometry. PI: proliferation index. **FIG. 5C and FIG. 5D**, Phenotype analysis performed after homeostatic proliferation. A significant higher percentage of cells previously treated with TWS119 maintained the preferred CD28⁺CD62L⁺ phenotype. **FIG. 5E**, After homeostatic proliferation, superior polyfunctionality is maintained in cells previously treated with TWS119. In contrast, cells untreated with TWS119 remain poorly polyfunctional.

[13] **FIGS. 6A-B**. Results of experiments demonstrating that TWS119 induces Wnt pathway activation in memory T cells after *ex vivo* activation. Memory T cells were isolated from fresh collected PBMCs and stimulated with plate-bound anti-CD3/CD28 for 2h or 6h, in the presence (triangle) and absence (half square) of 3mM TWS119. **FIG. 6A**, At 6h, intracellular level of β -catenin was measured by flow cytometry (3mM TWS119, blue; DMSO, red; isotype control, shaded grey). **FIG. 6B**, qPCR performed at 6h showed TWS119 treatment upregulated all five Wnt pathway target genes (solid triangle: TWS119; open square: DMSO).

[14] **FIG. 7A-C.** Results of experiments demonstrating that activation of Wnt signaling inhibits the overall expansion of influenza-specific cell but increases the tetramer specificity in culture. Human CD8⁺ T cells were stimulated with autologous, M1-peptide loaded moDCs for two weeks (stimulations were given on D0 and D7). The culture was also treated with different amount of TWS119, as indicated. Tetramer staining and cell counts were determined on D7 and D14. Cell numbers shown were presented based on a starting culture composed of 100,000 CD8⁺ T cells. **FIG. 7A and 7B**, Despite higher antigen specificity in the culture as shown in FIG. 2, TWS119 inhibits the expansion of all cells in the culture as well as the M1-specific cells being generated. **FIG. 7C**, CD8⁺ T cells were labeled with CFSE before second moDC stimulation. On D7, CFSE dilution was analyzed within CD8⁺M1-tetramer⁺ or CD8⁺M1-tetramer⁻ populations. TWS119 inhibited the overall proliferation of M1-specific T cells but more inhibitory effect is seen in non-specific CD8⁺ T cells. *: P value<0.05.

[15] **FIG. 8.** Results of experiments demonstrating that individual effector function changes in response to Wnt pathway activation in M1-specific T cells. On D14, after two M1-loaded moDC stimulations, expanded M1-specific cells were rechallenged with M1-loaded T2 cells for six hours to assess their polyfunctionality. IL-2 and TFN α production are significantly improved when cells were treated with TWS119. *: P value<0.05.

[16] **FIG. 9.** Results of experiments demonstrating that CMV p65-specific T cells are terminally differentiated memory cells. Freshly isolated CD8⁺ T cells from healthy donors were stained with anti-CD8 and M1- or CMV-tetramer along with other surface markers to determine the ex vivo phenotype of antigen-specific T cells. **FIG. 9A**, Majority of M1-specific cells was CD28⁺CD27⁺ but majority of pp65-specific cells was CD28⁻CD27⁻. **FIG. 9B**, Percentage of tetramer-specific cells within each memory differentiation subset according to the definition of Sallusto *et al.* (Sallusto *et al.*, 2004). M1-specific cells were either CCR7⁺CD45RA⁺ (T_N) or CCR7⁺CD45RA⁻ (T_{CM}) or CCR7⁻CD45RA⁻ (T_{EM}) phenotype. In contrast, the majority of CMV-pp65-specific cells were CCR7⁻CD45RA⁺ (T_{EMRA}) phenotype. *: P value<0.05.

Figures for Examples 9-17

[17] **FIG. 10.** Influenza virus M1-pulsed moDCs induce concentration-dependent proliferation of M1-specific CD8⁺ T cells with variable levels of polyfunctionality. (**FIGS.**

10A-C) Percentage of M1-specific T cells and M1-specific T cell expansion. Autologous moDCs pulsed with variable amounts of the HLA-A*0201 restricted immunodominant M1 peptide (10 μ M~10 fM) were used to stimulate HLA*A201+ CD8+ T cells ex vivo weekly for 2 weeks. Cultures were analyzed on D14. **(FIGS. 10D-H)** Functional assessment of M1-specific T cells: polyfunctionality assessment of M1-specific T cells reveals significant impairment of T cells induced with a high antigen concentration (10 μ M peptide-pulsed moDCs). **(FIG. 10D)** Top row: intracellular cytokine staining for IL-2 versus CD107. Numbers in red represent the percentage of IL-2–producing (right upper quadrant) versus IL-2–negative cells (right lower quadrant) in the CD107a-positive cell population. Bottom row: intracellular cytokine staining for TNF- α versus MIP-1 β . Numbers in red represent the percentage of TNF- α –producing(right upper quadrant) versus TNF- α –negative cells (right lower quadrant) in the MIP-1 β –positive cell population. **(FIG. 10E)** Percentage of individual effector function expression out of total antigen specific response. **(FIG. 10F)** Polyfunctionality pie charts. Each slice of pie represents the percentage of cells expressing between 1~5 effector functions. To compare different pie charts, permutation test with 10,000 repetitions was used. **(G)** The percentage of 5+ function cells in each T cell culture. **(FIG> 10H)** Amount of IL-2 produced by 5+ function cells. IL-2 production was quantified by measuring MFI and plotted for 5+ function cells. * $P < 0.05$. The data are representative of at least 3 assessments with 5 different donors.

[18] FIG. 11. Microarray analysis reveals unique molecular signatures associated with T cell polyfunctionality. **(FIG. 11A)** Heat map of differentially expressed genes among T cells induced by 10 μ M moDCs or 10 nM moDCs (adjusted $P < 0.05$, fold change > 1.6). Red and blue indicate increased and decreased gene expression, respectively. **(FIG. 11B)** Expression level of EOMES among T cells induced by 10M moDCs (red) or 10 nM moDCs (blue), determined by flow cytometry. **(FIG. 11C)** Real-time qPCR validation of TCF7 level. T cells induced with 10 μ M or 10 nM pulsed moDCs were tetramer sorted and analyzed by qPCR. * $P < 0.05$. (29. **(FIG. 11D)** GSEA shows that high antigen concentration–induced T cells are enriched with exhaustion signature from LCMV or HIV. In contrast, optimal concentration–induced T cells were enriched with the genetic signature of memory T cells. Molecular signature from PD-1 ligation was not able to distinguish between T cells stimulated with high and optimal antigen concentrations.

[19] FIG. 12. Polyfunctionality regulation by antigen concentration is independent of inhibitory receptor signaling. **(FIG. 12A)** On D14, T cells were harvested and stained with

anti-CD8 and M1 tetramer, then assayed for inhibitory receptor expression. Representative histograms shown were gated on CD8⁺, M1 tetramer⁺ population. Red: 10 μ M. Blue: 10 nM. Yellow: 10 fM. High antigen concentration–induced M1-specific T cells (10 μ M) showed higher inhibitory receptor expression except for BTLA. **(FIG. 12B)** Real-time qPCR analysis of BATF expression level in D14 sorted M1-specific cells. **(FIG. 12C)** Blockade of PD-1 signaling by anti–PD-1 antibody (clone EH12.2H7) during high antigen concentration stimulation did not improve polyfunctionality. The lack of any effect of anti–PD-1 treatment was seen at the standard dose used of 10 μ g/ml (see above) as well as at 10-fold higher concentrations of anti–PD-1 treatment. Isotype control antibody (clone MG1-45) was added at the same concentration. **(FIGS. 12D-F)** Ex vivo CD8⁺ T cells stimulated with different concentrations of aAPCs weekly for 2 weeks showed different levels of T cell polyfunctionality. aAPCs were made by conjugating biotinylated HLA-A2–M1 and biotinylated anti-CD28 complex onto anti-biotin microbeads (Miltenyi Biotec). **(FIG. 12D)** Percentage of tetramer-positive cells and **(FIG. 12E)** total M1-specific cell expansion. Higher concentrations of aAPCs induced more robust cell expansion, similar to results seen in FIG. 1 with peptide-pulsed moDC-induced T cells. **(FIG. 12F)** Polyfunctionality assessment of aAPC-stimulated M1-specific T cells. High concentration aAPCs induced M1-specific T cells with lower polyfunctionality even in the absence of inhibitory receptors on the aAPCs. The results are representative of 3 independent experiments performed on at least 3 different subjects. *P < 0.05.

[20] FIGS. 13A-D. MAPK/ERK pathway controls T cell polyfunctionality. **(FIG. 13A)** D14 tetramer–sorted high and optimal antigen concentration–stimulated T cells were restimulated with PMA/ionomycin for 10 minutes and analyzed for expression of pERK by flow cytometry. Red, high concentration; blue, optimal concentration; Gray, unstimulated. **(FIG. 13B)** ERK inhibitor U0126 inhibits pERK expression in a concentration-dependent fashion. T cells stimulated by the optimal concentration of antigen were restimulated with PMA/ionomycin in the presence or absence of varying concentrations of the ERK inhibitor U0126 for 10 minutes. Cells were then stained for pERK expression. Red, 100 μ M U0126; orange: 1 μ M U0126; blue, no U0126; gray, unstimulated. **(FIGS. 13C-D)** Optimal concentration–induced T cells were incubated with T2 target cells for 6 hours in the presence of various amounts of U0126 before intracellular cytokine staining and polyfunctionality analysis. U0126 selectively inhibited cytokine secretion but not CD107a upregulation and

MIP-1 expression. **(FIG. 13E)** T cells still mediated effective lysis of peptide-pulsed T2 target cells in the presence of 100 μ M U0126.

[21] FIG. 14. Upregulation of SPRY2 in high antigen concentration– induced T cells inhibits polyfunctionality. **(FIG. 14A)** qPCR comparison of SPRY2 expression levels on high and optimal antigen concentration–stimulated T cells. **(FIG. 14B)** The knockdown efficiency of lentiviral particles containing shRNA targeting SPRY2. Twenty- four hours after the second high antigen concentration moDC stimulation, cells were transduced with either SPRY2 knockdown virus or NT sequence virus. qPCR, flow cytometry staining of SPRY2 (red, NT virus control; blue, SPRY2 knockdown virus; gray, FMO control) and polyfunctionality assessment were performed on D14. **(FIG. 14C)** Representative flow plots of effector functions in virus-transduced T cells. T cells transduced with SPRY2 knockdown virus exhibit enhanced cytokine production of IL-2 and TNF- α as compared with control NT virus. SPRY2 knockdown had no impact on either CD107a upregulation or MIP-1 β production. **(FIG. 14D)** SPRY2 knockdown virus–transduced T cells showed greater percentage of 5+ polyfunctional T cells than control virus–transduced T cells. * $P < 0.05$. The data are representative of more than 3 independent experiments performed on 3 different subjects.

[22] FIGS. 15A-F. SPRY2 inhibition enhances HIV-specific T cell polyfunctionality. **(FIGS. 15A-B)** SPRY2 expression was studied in 12 HIV-infected HLA-A2+ patients. HIV-Gag-specific T cells and influenza M1-specific T cells were sorted by pentamer/tetramer staining and mRNA extracted from Gag- and M1-specific cells for qPCR analysis. **(FIG. 15A)** An example of flow cytometry–based simultaneous analysis of Gag- and M1-specific T cells from PBMCs of an HIV-infected donor. **(FIG. 15B)** qPCR and flow cytometry analysis of SPRY2 and PD-1 expression in Gag-specific, M1-specific, and total CD8+ T cells from HLA-A2+ HIV donors. Statistical analyses were performed by nonparametric Wilcoxon matched-pairs signed rank test. **(FIGS. 15C–F)** PBMCs from 19 HIV-infected patients were activated with soluble anti-CD3, anti-CD28, and a mix of CEF/HIV peptide pools. Some cultures were also treated with anti-PD-1 (10 μ g/ml) to block PD-1 signaling during activation. 24 hours later, cells were transduced with SPRY2 knockdown (KD) or NT control lentivirus. On D7 after virus transduction, PBMCs were stimulated with CEF or HIV Gag, Nef, and Tat peptide pools for 6 hours and analyzed for polyfunctionality. **(FIGS. 15C–E)** Inhibition of SPRY2 expression led to augmented HIV-specific T cell polyfunctionality above and beyond that seen by inhibition of PD-1 pathway

alone. (**FIG. 15F**) PD-1 blockade alone or in combination with SPRY2 inhibition had no significant effect on the CEF-specific response.

[23] **FIG. 16.** Polyfunctionality profile of T cells induced with different antigen concentrations. Complete polyfunctionality profile of M1-specific T cells on D14. For simplicity, only T cells induced with 10 μ M moDCs, 10nM moDCs, and 10fM moDCs are shown. 10 μ M moDCs induced T cells with a dominant subpopulation producing MIP-1 β and CD1-7a, but none of the tested cytokines (CD107a+, MIP-1 β +, IL-1-, TNF α -, IFN γ -). In contrast, 10nM moDCs induced highest percentage of cells capable of five effector functions simultaneously (CD107a+, MIP-1 β +, IL-2+, TNF α +, IFN γ +).

[24] **FIGS. 17A-B.** Highly polyfunctional T cells are equipped with superior proliferative capacity. T cells previously stimulated for 2 weeks with either high (10 μ M) or optimal (10 nM) antigen concentration-pulsed moDCs were re-stimulated with different concentrations of antigen-pulsed moDCs. After seven days, cells were harvested, counted, analyzed by tetramer staining and assayed for polyfunctionality. **FIG. 17A**, M1-tetramer staining, D21. **FIG. 17B**, absolute cell expansion after third stimulation. Despite various antigen concentration conditions used for third stimulation, T cells previously stimulated with optimal (10 nM) antigen concentration exhibited superior third week cell number expansion (for all restimulation doses, $P < .05$).

[25] **FIG. 18.** Naïve T cells exhibit impaired polyfunctional response in response to high concentration of anti-CD3/CD28 stimulations. Naïve T cells were isolated using Miltenyi naïve CD8+ T cell isolation kit. Cells were stimulated with plate-bound antibodies at the indicated concentrations (equal concentrations of anti-CD3, clone HIT31, and anti-CD28, clone 28.2). Cells were harvested on D7 and restimulated with PMH/ionomycin for 5 hours before staining for intracellular cytokines. Consistent with what was observed with M1-specific T cells, strong stimulation of naïve T cells induced poorly polyfunctional T cells.

[26] **FIG. 19.** Phenotypic characterization of M1-specific T cells induced by different antigen concentrations. On D14, T cells were harvested and stained with anti-CD8, M1 tetramer and differentiation markers (**FIG. 19A**) or transcription factors (**FIG. 19B**). Representative histograms shown were gated on CD8+, M1 tetramer+ population. Red, 10 μ M; blue 10 nM; yellow 10fM. All antigen concentrations induced M1-specific T cells with a CD45RO⁺, CCR α -, CD28+, CD27+ effector memory phenotype. High (10 μ M) antigen

concentration-induced M1-specific CD8⁺ T cells expressed significantly lower level of eomesodermin (**FIG. 19B**) but maintained high levels of T-bet and bcl-2.

[27] **FIG. 20.** Upregulation of Spry2 in high concentration antigen-induced cells depends on the prior activation of MAPK/ERK pathway. D7 CD8⁺ T cells were restimulated with either high (10 μ M) or optimal (10nM) concentration M1 peptide-pulsed moDCs in the presence of different amounts of the ERK inhibitor, U0126. On D14, T cells were harvested and analyzed for SPRY2 levels. **FIG. 20A**, CD8⁺ T cells induced with 10 μ M antigen were analyzed for SPRY2 levels after treatment with varying concentrations of U0126 (1 μ M green; 100 nM, blue; no U0126, red). SPRY2 levels in T cells induced with optimal concentration of antigen (10 nM, shaded teal). **FIG. 20B**, inhibition of ERK pathway during prior T cell activation significantly inhibited the subsequent upregulation of SPRY2 (*P value <0.05).

[28] **FIG. 21.** Representative HIV-specific T cell polyfunctional response and *ex vivo* polyfunctionality. PBMCs from HIV-infected patients were thawed, rested overnight and stimulated with peptide pools for six hours prior to cytokine analysis. **FIG. 20A**, the direct *ex vivo* Gag and CEF-specific polyfunctional responses. Gag-specific T cells exhibit much a much lower level of polyfunctionality. **FIG. 20B**, representative flow cytometry results of Gag-specific polyfunctional response in anti-PD-1 and *SPRY2*-knockdown virus treated cells.

Table Legends

[29] **Supplementary Table 1.** Functional clustering of differentially expressed genes between high (10 μ M moDCs) and optimal concentration (10 nM moDCs)-induced T cells (P<0.05, fold change >1.6). Functional clustering was performed by DAVID/BP-FAT platform. For simplicity, only certain functional groups highly related to immune response are shown. Red and blue colored numbers indicate gene expression fold change as compared between different concentration-induced T cells. Refer to Table 1 for a full list of differentially expressed genes and Supplementary Table 2 for a full list of functional groups.

[30] **Supplementary Table 2.** Full list of differentially expressed genes identified by the microarray experiment. Group A, high antigen concentration (10 μ M); group B, optimal antigen concentration (10 nM).

[31] **Supplementary Table 3.** A full list of functional groups generated from the full list of differentially expressed genes by GO-BP-FAT (Gene Ontology Biological Process, FAT term) tool on the DAVID platform.

DETAILED DESCRIPTION

[32] Human T cells stimulated by a high concentration of antigen lack polyfunctionality and express a transcription profile similar to exhausted T cells. Several specific pathways were implicated by the transcription profile in control of T cell polyfunctionality.

MAPK/ERK pathway

[33] As described below, we investigated the control of polyfunctionality in primary human virus-specific CD8⁺ T cells in response to antigen. In our model system, memory influenza-specific T cells repetitively stimulated with a high antigen concentration, 10 μ M of the influenza M1 peptide on monocyte-derived DCs (moDCs), showed robust influenza antigen-specific CD8⁺ T cell proliferation, but low levels of polyfunctionality. In contrast, an optimal antigen concentration was determined, which induced highly polyfunctional influenza-specific T cells. An antigen concentration-dependent effect on polyfunctionality could also be demonstrated in naive human CD8⁺ T cells. Genomic gene set enrichment analysis (GSEA) revealed that the global transcriptome of high antigen concentration-induced T cells was similar, but not identical, to that of exhausted T cells observed in chronic infections. Importantly, low-level polyfunctionality induced by high antigen concentration stimulation led to increased expression of inhibitory receptors without evidence of inhibitory receptor signaling. Genetic and biochemical studies indicated that high antigen concentration impaired CD8⁺ T cell polyfunctionality through inhibition of the MAPK/ERK pathway via upregulation of sprouty-2 (*SPRY2*), a negative regulator of the MAPK/ERK pathway. Based on these findings, we analyzed a cohort of HIV-infected patients and found that *SPRY2* is involved in HIV-specific T cell exhaustion. HIV-specific T cells showed marked increased levels of *SPRY2* mRNA and *SPRY2* protein compared with influenza-specific T cells from the same donors. Furthermore, shRNA-mediated inhibition of *SPRY2* enhanced HIV-specific polyfunctionality independently of PD-1 blockade. When shRNA-mediated inhibition of *SPRY2* was analyzed in the presence of anti-PD-1, HIV Gag-specific CTL had levels of polyfunctionality similar to the non-

exhausted CEF responses. *SPRY2* thus appears to mediate inhibition of HIV-specific T cell polyfunctionality independently of the PD-1 pathway. These findings advance our understanding of the molecular control of T cell polyfunctionality and indicate what we believe to be a novel therapeutic target to reverse T cell dysfunction associated with chronic viral infection or cancer.

[34] T cells stimulated with a high concentration of antigen upregulated sprouty-2 (*SPRY2*), a negative regulator of the MAPK/ERK pathway. The clinical relevance of *SPRY2* was confirmed by examining *SPRY2* expression in HIV-specific T cells, where high levels of *SPRY2* were seen in HIV-specific T cells and inhibition of *SPRY2* expression enhanced the HIV-specific polyfunctional response. Our findings indicate that increased *SPRY2* expression during chronic viral infection reduces T cell polyfunctionality and identify *SPRY2* as a potential target for immunotherapy.

Decreasing SPRY2 Expression

[35] Spry2 expression can be inhibited by any method known in the art. In some embodiments, Spry2 expression is inhibited by a Spry2-targeted ribozyme, a Spry2-targeted antisense oligonucleotide, or a Spry2-targeted siRNA. In some embodiments, Spry2 expression is inhibited by an aptamer, a protein, a peptide, a cyclic peptide, a peptidomimetic or a small molecule. In some embodiments, Spry2 expression is inhibited by a polypeptide fragment or mutant form of a Spry2 protein, which may function as a competitive inhibitor of a Spry2-interacting polypeptide (*e.g.*, the c-Cbl E3 ubiquitin ligase, EGFR, Ras, or CIN85). *See, e.g.*, US 2007/0066522.

Wnt signaling pathway

[36] A second pathway identified was the Wnt signaling pathway. The Wnt signaling pathway was identified in the microarray pathway analysis suggesting it may be involved in polyfunctionality regulation.

[37] Wnt signaling pathway has been extensively studied in embryonic development (MacDonald *et al.*, 2009) and regulation of self-renewal of pluripotent stem cells (Kalani *et al.*, 2008; Zeng *et al.*, 2005). Earlier reports of the importance of Wnt signaling in the immune system comes from studies in the context of T cell development in

the thymus (Fleming *et al.*, 2008; Schilham *et al.*, 1998; Staal *et al.*, 2008). More recently, accumulating studies indicate that Wnt pathway also controls peripheral immune responses. For example, Wnt pathway activation inhibits naïve T cell proliferation and effector differentiation, preferentially generating a novel T cell subset with stem-cell features (TSCM, T memory stem cells) (Gattinoni *et al.*, 2009), was preferentially generated. Compared with other memory subsets, TSCM cells lack immediate effector functions but are multipotent and have enhanced self-renewal capacity to persist as memory cells *in vivo* (Gattinoni *et al.*, 2011; Gattinoni *et al.*, 2009; Lugli *et al.*, 2013). Other roles of Wnt signaling in mediating mature T cell response have also been reported. TCF1 is essential for generating memory CD8⁺ T cells (Jeannet *et al.*, 2010). Enforced expression of β -catenin and TCF1 inhibits the effector phase of the immune response and enhances the generation of memory T cells in response to *Listeria monocytogenes* infection in mice (Zhao *et al.*, 2009). Wnt signaling activation also arrests the naïve to effector differentiation in human peripheral and cord blood-derived T cells (Muralidharan *et al.*, 2011).

[38] We found that during antigen-driven expansion of human virus-specific T cells, Wnt pathway activation enhances the CD62L⁺, CD28⁺, and KLRG1⁻ central memory T cell phenotype. This effect is novel and distinct from the effects of Wnt pathway on naïve T cells and not predicted from that previous work. Such effects are not only seen in influenza-specific response but can also be seen in dysfunctional HIV-specific T cells. These findings provide the first evidence that dysfunctional virus-specific human T cell response could be chemically reprogrammed to polyfunctionality and have strong implications for chronic viral infections and cancer.

[39] The ability to artificially change the program of somatic cell differentiation and function has been gradually revealed in the past decade. Mounting evidence, including this current report, suggests that Wnt pathway controls cell fate in various tissues beyond the stage of embryonic stem cells. For example, Wnt pathway activation promotes epithelial-mesenchymal transdifferentiation (EMT) (Anson *et al.*; Wu *et al.*), cardiac hypertrophy (Gessert and Kuhl) and wound healing (Whyte *et al.*). In the immune system, forced expression of Wnt pathway proteins are associated superior memory T cell formation (Jeannet *et al.*, 2010; Zhao *et al.*, 2009; Zhou *et al.*, 2010) while Wnt pathway negatively regulates regulatory T cell functions (van Loosdregt *et al.*, 2013). Activation of Wnt pathway

also programs dendritic cells and NKT cells to tolerance induction (Deng *et al.*; Oderup *et al.*).

[40] In this disclosure, we demonstrate that enhancing Wnt signaling promotes the reprogramming of human virus-specific T cells to a polyfunctional status associated with many other stemness features. Polyfunctionality is a feature of early memory T cells. Such finding, nevertheless, contrasts the results obtained from naïve T cells. When Wnt pathway activation is activated in naïve T cells, a population of naïve-like, T_{SCM} cells (CD45RO-CCR7+CD45RA+CD62L+CD27+CD28+ IL7R α +CD95+) is generated (Gattinoni *et al.*, 2011). TSCM cells have limited effector functions but TWS119-treated memory T cells have substantially better polyfunctionality. This discrepancy could be explained by the fact that, the majority of virus-specific precursor cells in healthy donors have already been exposed to antigen and thus have acquired most, if not all effector functions. Despite T_{SCM} cells are shown to be superior to central memory cells for adoptive therapy against cancer in preclinical mice models (Gattinoni *et al.*, 2011), the number of T_{SCM} cells from human peripheral blood is extremely limited, thus limiting its potential in clinical use.

[41] We have also found that Wnt pathway activation enhanced the expression of CD62L on T cells. Increased expression of CD62L is an important characteristic since CD62L+ T cells are capable of entering secondary lymphoid organ and are superior in controlling pathogen challenge (Gattinoni *et al.*, 2005; Hengel *et al.*, 2003). TWS119-treated cells also express higher level of CD28 and lower level of KLRG1, again indicating those cells are of less-differentiated state and are preferable for adoptive immunotherapy {Klebanoff, 2012 #588; Ebert, 2012 #603}.

[42] The feasibility of reprogramming terminal differentiated T cells offers many practical implications. Because TWS119-treated cells express many central memory T cell associated stemness features, we believe these cells are of superior quality for adoptive T cell therapy. Since the numbers of donor-derived moDCs are very limiting clinically, large number of polyfunctional T cells could be potentially achieved by combining aAPC technology with TWS119. Finally, the enhancement of polyfunctionality could provide a therapeutic possibility for curing chronic virus infections.

[43] This disclosure describes the impact of Wnt pathway activation on polyfunctionality in memory T cells. Unexpectedly, instead of limiting T cell effector

functions (as happens in naïve T cells), Wnt pathway activation dramatically enhances memory T cell polyfunctionality. This effect not only is observed with the less-differentiated memory T cell specific for influenza virus, but also occurs in the terminally differentiated CMV virus-specific response. Importantly, the effect of TWS119 does not result from cell proliferation arrest but can be observed in cells already underwent multiple divisions. Moreover, the TWS119-treated cells exhibit many features of “stemness” because treated cells are capable of maintaining the CD62L⁺ polyfunctional phenotype during homeostatic proliferation and express higher level of anti-apoptotic proteins. These results provide strong evidence that polyfunctionality reprogramming in memory cells can be achieved with Wnt pathway activation. Finally, using the acellular aAPCs, we demonstrated that combining aAPC technology with Wnt pathway modulation is a promising strategy to induce large number of highly functional human antigen-specific T cells for adoptive immunotherapy.

Activators of the Wnt/ β -Catenin Pathway

[44] The Wnt/ β -catenin pathway can be activated by any method known in the art. Activators of the Wnt/ β -catenin pathway are disclosed, *e.g.*, in Table 1 of US 2012/0046242 and in US 2013/0143227. Examples of Wnt signaling pathway activators include Wnt1, Wnt2, Wnt2b, Wnt3, Wnt3a, Wnt4, Wnt5a, Wnt5b, Wnt6, Wnt7a, Wnt7b, Wnt8a, Wnt8b, Wnt9a, Wnt9b, Wnt10a, Wnt10b, Wnt11, and Wnt16b. In some embodiments, the Wnt/ β -catenin pathway is activated by inhibiting serine-threonine kinase glycogen synthase kinase-3 β (GSK3); *see, e.g.*, US 2001/0052137. Inhibitors of GSK3 include lithium, LiCl, bivalent zinc, BIO, SB216763, SB415286, CHIR99021, QS11 hydrate, TWS119, Kenpaullone, alsterpaullone, indirubin-3'-oxime, TDZD-8 and Ro 31-8220 methanesulfonate salt; Axin inhibitors, APC inhibitors, norrin, and R-spondin 2; *see* US 2011/0223660.

Pharmaceutical Compositions and Therapeutic Methods

[45] An inhibitor of SPRY2 expression and/or an activator of the Wnt/ β -catenin pathway can be formulated in a composition suitable for therapeutic administration to treat chronic infections (*e.g.*, HIV, HCV) and cancer. Such a pharmaceutical composition may consist of the active ingredient alone, in a form suitable for administration to a subject, or the pharmaceutical composition may comprise the inhibitor of SPRY2 expression and/or an activator of the Wnt/ β -catenin pathway and one or more pharmaceutically acceptable carriers, one or more additional ingredients, or some combination of these.

EXAMPLE 1. Methods for Examples 2-8

[46] Study subjects and cell isolation. Fresh human CD14⁺ and CD8⁺ cells were isolated from PBMCs of seven HLA*0201 positive healthy donors using Miltenyi isolation kits. Monocyte-derived dendritic cells (moDCs) were generated as previously described (Ndhlovu *et al.*, 2010; Oelke *et al.*, 2003). Briefly, CD14⁺ cells were cultured in the presence of 50ng/ml IL-4 and 100ng/ml GM-CSF for six days and then matured with 10ng/ml TNF α , 10ng/ml IL-1, 1000U/ml IL-6 and 1 μ g/ml prostaglandin E2.

[47] Antigens-specific cell activation and expansion by moDCs or aAPCs. moDCs were pulsed with 1 μ g/ml M1 (58-66; GILGFVFTL, SEQ ID NO:1) or CMV pp65 (NLVPMVATV; SEQ ID NO:2) peptide for an hour at 37°C. Peptide-loaded aAPCs were produced as previously described (Chiu *et al.*, 2011; Oelke *et al.*, 2003). For each stimulation, 1 million peptide-pulsed moDCs or 3 million aAPCs were cocultured with 3 million CD8⁺ cells in a 96 well, U-bottom plate. The culture media was complete RPMI media supplemented with antibiotics, 5% autologous plasma, and 3% T cell factor (Oelke *et al.*, 2003).

[48] Antibodies and flow cytometry. The following antibodies were used: anti-CD8 pacific blue (Biolegend, HIT8a), anti-CCR7 PE (Biolegend, G043H7), anti-CD45RA APC (Biolegend, HI100), anti-CD62L Alexa 647 (Biolegend, DREG-56), anti-CD27 Pe-Cy7 (Biolegend, M-T271), anti-CD28 pacific blue (Biolegend, CD28.2), anti-IL-2 Percp-Cy5.5 (MQ1-17H12), anti-MIP-1 β PE (BD, D21-1351), anti-TNF α Pe-Cy7 (BD, Mab11), anti-IFN γ APC (BD, 25723.11), anti-CD107a FITC (BD, H4A3), anti-IL15Ra APC (Biolegend, JM7A4), anti-CD122 BV421 (Biolegend, TU27), anti-CD132 APC (Biolegend, TUGh4). Alexa647-conjugated anti-KLRG1 antibody (Clone 13F12F2) was kindly provided by professor Hans-Peter Pircher (University of Freiburg, Germany). Intracellular staining for β -catenin was performed using alexa488-conjugated anti- β -catenin antibody (eBioscience, clone 15B8) according to the manufacturer's protocol.

[49] Intracellular cytokine detection and polyfunctionality analyses. 3×10^5 M1 or CMV-specific CD8⁺ T cells were incubated with 1.5×10^5 target cell (HLA*0201 positive T2 cell line) at 37°C in the presence of monensin, brefeldin A and anti-CD28/CD49d costimulation (all from BD Biosciences). For experiments about CD107a, Anti-CD107a was added at the start of stimulation. Target T2 cells were loaded with M1 peptide at a

concentration of 1 μ M for at least an hour and washed extensively before use. Unloaded target cells were used as background stimulation. After 6 hours, cells were washed twice with FACS wash buffer and then stained with viability dye, anti-CD3 and anti-CD8 for 20 minutes. Cells were then fixed and permeabilized at 4°C with the Cytofix/Cytoperm kit (BD Biosciences) following the manufacturer's protocol. A cocktail of fluorophore-conjugated antibodies containing anti-IL-2, anti-TNF α , anti-IFN γ and anti-MIP-1 β were added to the cells and stained for two hours. Subsequently cells were washed twice before acquisition by a LSRII flow cytometer. The polyfunctionality analysis was performed by the Boolean gate platform of FlowJo version 9.3.1 software (TreeStar, Ashland, Oregon).

[50] Cell proliferation analyses. T cells were labeled with 1mM Carboxyfluorescein diacetate Succinimidyl Ester (CFSE, Invitrogen, C34554) in culture media at 37°C for 10 minutes. CFSE were quenched by adding 5 times more cold media, washed twice and let sit at 37°C for an additional 30 minutes. After one more wash with medium cells were then cocultured with moDCs. On selected days as indicated, cells were harvested for tetramer staining and other assays. After gated on CD8+, tetramer-specific cells, the progressive dilution of CFSE corresponding to cell division cycles was analyzed on the Flowjo's proliferation platform. Proliferation index (P.I.) was calculated as the total number of cell divisions divided by the number of cells that have divided.

[51] Quantitative RT-PCR. Cells were lysed with Trizol and stored at -80°C. cDNA synthesis was performed by using Taqman Gold RT-PCR kit. Measurements of mRNA level of Wnt target genes (Tcf7, Lef1, Nlk, Fzd7 and Jun) were done by Real-time qPCR (Taqman gene expression assay) using Taqman gene expression kits. *18S* was used as internal controls to determine the differential mRNA expression between samples.

[52] Statistical Analyses. Results were shown as mean $\pm$ SD. Student's *t* test was used to determine the statistical significance of difference between treatment conditions. For comparison between pie charts, permutation test was performed using SPICE (Data Mining & Visualization Software for Multicolor Flow Cytometry) version 5.2. (NIAID, NIH, available at exon.niaid.nih.gov/)(Roederer *et al.*, 2011).

EXAMPLE 2. TWS119 has distinct effect on polyfunctionality in memory versus naïve T cells.

[53] In the literature, a GSK3 β (serine-threonine kinase glycogen synthase kinase-3 β) inhibitor, TWS119, has been widely used to mimic the activation of canonical Wnt-signaling pathway (Forget *et al.*, 2012; Gattinoni *et al.*, 2011; Gattinoni *et al.*, 2009; Muralidharan *et al.*, 2011; Staal *et al.*, 2008). Inhibition of GSK3 β prevents the degradation of β -catenin promotes the translocation of β -catenin into the nucleus where it binds to other transcription factors that promote the transcriptional activities of Wnt target genes. Upon TWS119 treatment, all the Wnt target genes tested were upregulated in memory T cells (**FIG. 6**).

[54] TWS119 treatment of naïve T cells inhibits their acquisition of effector functions (Gattinoni *et al.*, 2009). To determine the effect of Wnt pathway activation on memory T cell function, we isolated naïve and memory CD8⁺ T cells and activated them with anti-CD3/CD28 in the presence of TWS119. The effector functions were studied on D7. As shown in **FIG. 1A**, effector functions of naïve T cells were severely impaired by TWS119. In contrast, memory T cells activated in the presence of TWS119 expressed much higher level of IFN γ and TNF α . In fact, among memory T cells, all the cytokines tested including IL-2, TNF α and IFN γ as well as the polyfunctionality (as determined by the percentage of cell making all three cytokine simultaneously), were significantly upregulated by TWS119 treatment (**FIG. 1C**). This is in sharp contrast to what is observed in naïve T cells, in which all the effector functions and polyfunctionality were inhibited by TWS119 (**FIG. 1B**).

[55] To determine if the impact on memory T cell polyfunctionality by TWS119 was driven by a specific memory subset, we sorted cells from individual memory subset (T_{CM}: CCR7⁺CD45RA⁻; T_{EM}: CCR7⁻CD45RA⁻; T_{EMRA}: CCR7⁻CD45RA⁺) and activated them in the presence and absence of TWS119. We observed significant improvement in polyfunctionality with TWS119 treatment in all three sorted memory subsets, indicating that Wnt pathway activation affects all the subsets similarly.

[56] Because TWS119 also affects other signaling pathways, we examined the effect of other Wnt pathway modulators on polyfunctionality. Induction of polyfunctionality can be achieved by using another Wnt pathway activator, SKL2001 (**FIG. 1E**). Furthermore,

when AVN361 (a small molecule that inhibits β -catenin downstream of TWS119) was added to TWS119-treated culture, the induction of polyfunctionality was largely inhibited.

EXAMPLE 3. Wnt pathway activation increases the antigen specificity of human T cell culture during *ex vivo* expansion

[57] We next tested whether if Wnt pathway activation will affect the polyfunctionality of antigen-specific memory T cells. Autologous CD8⁺ T cells were stimulated with M1 peptide-pulsed monocyte-derived dendritic cells (moDCs) in the presence of different concentration of TWS119 weekly for two weeks to test the effect of TWS119 on polyfunctionality. First we determined the percentage of M1-specific T cells on D7 and D14. As shown in **FIG. 2A** and **FIG. 2B**, a higher percentage of M1-specific T cells was induced in the cultures treated with TWS119. However, TWS119 treatment impaired the expansion of all cells in the culture as well as the total number of M1-specific cells (**FIGS. 7A-B**). This effect was due to the fact that proliferation of non-specific cells was arrested in TWS119 treated cultures (**FIG. S3C**). Overall, TWS119 inhibition of M1-specific cell expansion was dose-dependent, but substantial proliferation was observed even with the highest dose tested (3mM).

EXAMPLE 4. Influenza M1-specific T cells expanded in the presence of Wnt pathway activation exhibit higher CD62L and better polyfunctionality

[58] To study the effect of TWS119 in an antigen-specific fashion, expanded M1-specific cells were rechallenged with M1-peptide-pulsed target cells and investigated for their ability to upregulate various effector functions (IL-2, TNF α , INF γ , CD107a and MIP-1 β). Expression of IL-2 is a feature early memory T cells and the first function to be lost when virus-specific T cells are repetitively stimulated to terminal differentiation or exhaustion (Wherry, 2011). The co-expression of these five effector functions, or polyfunctionality, is critical for optimal immunity against pathogens and correlates with memory T cell formation (Betts *et al.*, 2006; Joshi and Kaech, 2008; Seder *et al.*, 2008; Wherry, 2011). Compared with T cells expanded without TWS119, cells generated in the presence of 3mM TWS119 produced more IL-2 and TNF α when rechallenged with antigen (**FIG. 2C**). In contrast, the expression level of INF γ , CD107a and MIP-1 β were not changed by Wnt pathway activation (**FIG. 8**). When co-expression of five effector functions were analyzed, highest dose of TWS119 induced 28% of responding cells capable of executing five effector function

simultaneously (IL-2+TNF α +INF γ +CD107a+MIP-1 β +; “5+”), as opposed to only 9% in cells expanded without TWS119 treatment (**FIG. 2D**). TWS119 also enhanced the absolute number of 5+ polyfunctional cell being generated (**FIG. 2E**).

[59] We next performed phenotypic analysis on the expanded M1-specific cells. Consistent with previous studies (Gattinoni *et al.*, 2011; Muralidharan *et al.*, 2011), CD62L expression on M1-specific cells increased under TWS119 treatment. However, TWS119 also induced higher level of CD28 surface expression and lower level of KLRG1 expression (**FIG. 2F**). KLRG1 signaling has been shown to inhibit Akt phosphorylation and is associated with immunosenescence of T cells (Henson *et al.*, 2009).

EXAMPLE 5. Wnt pathway activation increases CMV-pp65-specific T cell polyfunctionality

[60] Influenza-specific T cells are known to be in early memory differentiation. T cells specific for chronic infections are constantly exposed to antigen stimulation and frequently persist *in vivo* as advanced differentiated cells. To determine if Wnt pathway activation can also reprogram virus-specific memory T cells with advanced differentiation to a polyfunctional state, we tested the effect of TWS119 on CMV pp65-specific T cells. In humans, CMV-specific T cells are known to be highly-differentiated, have limited effector functions and proliferative capability, and are constantly driven to a immunosenescence state (Fletcher *et al.*, 2005; Hertoghs *et al.*; Mekker *et al.*, 2012). When analyzed directly *ex vivo*, CMV-specific T cells have low levels of CD28 and CD27 and the majority of them are of the CD45RA+CCR7- phenotype (terminal effector, T_{EMRA}) (**FIG. 9**). Consistent with the terminal differentiation phenotype, upon stimulation with pp65 peptide, they produce little IL-2 and thus are not polyfunctional (**FIG. 3A**).

[61] To show that Wnt pathway activation could also modulate CMV-specific T cell polyfunctionality, CMV-pp65-specific T cells from healthy donors were expanded with autologous moDCs in the presence or absence of TWS119. After two weeks of pp65-pulsed moDCs stimulation, similar to what has been observed in the influenza-M1 response, TWS119-treated culture also had substantially improved CMV-pp65-specificity in culture (**FIG. 3B**). CMV-specific cells expanded without TWS119 still do not produce significant amount of IL-2. In sharp contrast, CMV-specific cells are reprogrammed to a much more polyfunctional state with TWS119 treatment and express higher level of CD62L and CD28

(FIGS. 3C-3E). These results indicate that highly differentiated, poorly polyfunctional CMV-specific T cells can also be reprogrammed to a polyfunctional state by Wnt pathway activation.

EXAMPLE 6. Effect of Wnt pathway activation on polyfunctionality and CD62L expression are seen in dividing cells

[62] Previous studies on TWS119 effect on naïve T cell differentiation were questioned on whether such effect is largely due to arresting cellular proliferation (Prlic and Bevan, 2011). In fact, naïve cells treated with the same dose of TWS119 (3mM) in a previous study were largely undivided (Muralidharan *et al.*, 2011). To further clarify if Wnt pathway activation only improves polyfunctionality in cells did not proliferate, we labeled M1-specific T cells with CFSE on D7 before restimulation and followed the expression of CD62L and polyfunctionality among different division generations on D14. As shown in FIG. 4A, the enhancement of CD62L and effector function expression in TWS119-treated cells clearly was seen even in cells that underwent a significant number of divisions.

EXAMPLE 7. The effect of Wnt pathway activation on polyfunctionality is not dependent on dendritic cells

[63] To test whether TWS119 acts directly on the T cells or rather through modulation of the stimulatory capacity of the dendritic cells, we tested the impact of TWS119 on polyfunctionality in a dendritic cell-free system. We used our previously described, HLA-Ig-based aAPCs to stimulate CD8⁺ T cells (Chiu *et al.*, 2011; Oelke *et al.*, 2003). Briefly, Briefly, HLA-A2-Ig and anti-CD28 were coupled to cell-sized (4.5μM) dextran-coated particles and loaded with either Flu-M1 or CMV-pp65-NLV peptide to generate aAPCs for CD8 T cell stimulation. As shown in FIGS. 4C-D, TWS119 significantly increased the antigenic specificity of the culture similarly to what had been observed with stimulation of moDCs. TWS119 also enhanced the polyfunctionality of both M1 and CMV-specific cells. Thus the effect of TWS119 on polyfunctionality does not seem to be dependent on dendritic cells and is largely intrinsic to T cells.

EXAMPLE 8. Wnt pathway activation-induced polyfunctionality is associated with many stemness features

[64] Finally, we sought to determine if Wnt pathway-induced highly polyfunctional T cells will be equipped with stem cell-like properties suggestive of superior survival. First we studied the expression level of anti-apoptotic proteins, Bcl-2 and Bcl-XL, in expanded M1-specific T cells. Bcl-2 and Bcl-XL overexpression has been shown to increase human embryonic stem cell expansion and survival (Bai *et al.*; Goff *et al.*). As shown in **FIG. 5A**, M1-specific T cells expanded in the presence of TWS119 expressed higher level of Bcl-2 and Bcl-XL. Similar results were seen with CMV-specific cells. In addition to superior survival, stem cells are characterized by their ability to self-renewal. Thus if TWS119-treated cells are reprogrammed to polyfunctionality, the polyfunctional phenotype should be maintained even after the treatment was stopped. We thus harvested TWS119-treated or non-treated M1-specific cells on D14 and further cultured these cells in the presence of IL-15 for another week (without TWS119). As shown in **FIG. 5B**, we found that TWS119-treated cells proliferated better than untreated cells. In addition, T cells previously treated with TWS119 also maintained higher CD62L and CD28 expression after homeostatic proliferation (**FIG. 5C**, **FIG. 5D**). Finally, cells previously treated with TWS119 still exhibited higher polyfunctionality after homeostatic proliferation (**FIG. 5E**). These results indicate that Wnt pathway activation by Wnt pathway activation reprograms human virus-specific T cells to a long-standing, young, polyfunctional phenotype.

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EXAMPLE 9. Methods for Examples 10-17

[65] Study subjects and purification of primary human PBMCs and CD14+ and CD8+ T cells. PBMCs from 5 healthy HLA*0201–positive donors and 15 HIV-infected patients were isolated by Ficoll-Paque PLUS gradient centrifugation following the manufacturer’s protocol (GE Healthcare). The HIV-infected, HAART-treated aviremic patients were recruited from Johns Hopkins and Case Western Reserve University. The average age of donors was 49 years old. For some experiments, CD14+ and CD8+ T cells were further purified from fresh PBMCs using the CD14+ cell–positive selection and CD8+ T cell–negative selection kits according to the manufacturer’s instructions (Miltenyi Biotec).

[66] Generation of peptide-loaded moDCs and aAPCs. The generation of moDCs was done by following the standard protocol as previously described (28,43). Briefly, CD14+ cells were cultured in complete RPMI supplemented with 5% autologous plasma, 100 ng/ml human granulocyte-macrophage colony stimulating factor, and 50 ng/ml IL-4. After 6 days of culture, a maturation cocktail containing 10 ng/ml TNF- α , 10 ng/ml IL-1, 1000 U/ml IL-6 and 1 μ g/ml prostaglandin E2 was added to culture. MoDCs were harvested on D7, and the mature DC phenotype was confirmed by flow cytometric analysis of CD80, CD86, and HLA-DR. moDCs were then pulsed with influenza virus matrix peptide M1 (58–66; GILGFVFTL; SEQ ID NO:1) at different concentrations in serum-free media for an hour at 37°C. Peptide-pulsed moDCs were then washed extensively to remove free peptide in solution before use. For generation of aAPCs, M1 peptide-loaded HLA-A2–Ig and anti-CD28 (clone 9.3) were biotinylated and coupled to anti-biotin coated microbeads (Miltenyi Biotec) and stored at 4°C (29) before use.

[67] *In vitro* expansion of M1-specific CD8+ T cells. Three million freshly isolated CD8+ T cells were co-cultured with 1 million moDCs in complete RPMI medium containing 5% autologous plasma, 3% T cell growth factor (28) and antibiotics. For micro-aAPC stimulation, different amounts of microaAPCs were co-cultured with CD8+ T cells. On D7 and weekly thereafter, T cells were harvested, counted, and replated at the same T cell/APC

density. Influenza M1-antigen specificity was determined by using HLA-M1-specific A*0201 PE or APC tetramers (GILGFVFTL; SEQ ID NO:1, Beckman Coulter).

[68] Detection of intracellular cytokines to assess polyfunctionality. For experiments involving M1-specific T cells, 2×10^5 CD8⁺ T cells were incubated with 1×10^5 target cells (HLA*0201-positive T2 cell line at 37°C in the presence of monensin, brefeldin A and anti-CD28/CD49d costimulation (all from BD Biosciences). Anti-CD107a was added at the start of stimulation. Target cells were pulsed with M1 peptide at a concentration of 1 μ M for an hour and washed twice before use. Unpulsed target cells were used as background stimulation. After 6 hours, cells were washed twice with FACS wash buffer and then stained with viability dye, anti-CD3, and anti-CD8 for 20 minutes. Cells were then fixed and permeabilized with the Cytofix/Cytoperm kit (BD Biosciences) following the manufacturer's protocol. A cocktail of fluorophore-conjugated antibodies containing anti-IL-2, anti-TNF- α , anti-IFN- γ , and anti-MIP-1 β was added to the cells and stained for an hour. For experiments involving HIV-specific T cell responses, 2×10^5 PBMCs were stimulated with different peptide pools at a concentration of 0.5 μ g/ml per peptide (Gag, Nef, Tat peptide pools, NIH AIDS reagent program; CEF peptide pool, Anaspec) for 6 hours for effector function detection. Complete combinations of different effector function were performed using the Boolean gate platform of FlowJo version 9.3.1 software (TreeStar). Reported data were further adjusted by background subtraction. Polyfunctionality pie charts were generated by SPICE (Data Mining and Visualization Software for Multicolor Flow Cytometry) version 5.2. (National Institute of Allergy and Infectious Diseases [NIAID], NIH) (44).

[69] Microarray experiment, data analysis, and qPCR validation. More than half a million M1-specific T cells induced by 10 μ M or 10 nM moDCs from 5 experiments on 3 donors were tetramer sorted and preserved in TRI reagent (Molecular Research Center) at -80°C. RNA extraction and microarray experiments were performed by Miltenyi Biotec. Hybridization was performed with single-colored Whole Human Genome 8 $\times$ 60K OligoMicroarrays (Agilent Technologies). Gene expression data were analyzed using Partek Genomic Suite. Each sample was normalized and log2 transformed before being compared by *t* test ($P < 0.05$). Genes with fold changes of more than 1.6 were selected for further analysis. Differentially expressed genes were functionally grouped and annotated using DAVID Bioinformatics Resources 6.7 (NIAID, NIH; available at david.abcc.ncifcrf.gov/home.jsp) (45). Functional annotation and pathway identification were performed using the Gene Ontology (GO) fat category, as provided by the DAVID platform.

Validations of genes were done by real-time qPCR(TaqMan gene expression assay) using *ACTB* or *HPRT1* as internal controls. The complete microarray dataset is available from GEO (GSE39761). *GSEA*. GSEA (46) was used to identify gene sets that are significantly enriched in either high concentration (10 μ M) or optimal concentration(10 nM) stimulation. The input to GSEA is the log2 transformed expression ratio for each gene. When expression of one gene was measured by multiple probes, we used the maximum expression ratio of all probes for that gene. GSEA (46) calculates a running sum of the statistic for each gene set. The test statistic is the maximum of the running sum (enrichment score), and permutation tests are performed to evaluate the empirical *P* value of the enrichment. Gene sets for LCMV exhaustion versus normal effector and memory T cells (25) were provided by John Wherry. Gene sets for HIV progressor and PD-1 signaling (26) were provided by Nicolas Haining.

[70] pERK staining and cytotoxicity assay. pERK detection was based on protocols as previously described (47). Briefly, M1-specific T cells were stimulated with PMA/ionomycin for 10 minutes before fixation with 2% formaldehyde and permeabilized with 90% methanol. Staining was performed with anti-phospho-p44/42 MAPK (ERK1/2) antibody from Cell Signaling(clone E10). Flow cytometry-based cytotoxicity assay (48) was used to investigate the effect of ERK inhibition on T cell-mediated HLA-A2-positive T2 cell killing.

[71] Production of shRNA-containing lentiviral particles and T cell transduction. shRNA sequence (5'-CTGAACAGAGACTGCTAGGATCATCCTTC-3'; SEQ ID NO:3) targeting the human *SPRY2* gene was cloned into pLKO.1 puro plasmid (provided by Joel Pomerantz. Viral particles were made by co-transfecting 20 ng of the PLKO plasmid plus 15 ng delta 8.9 plasmid and 10 ng pCMV-VSV-G into HEK293T cells (plated 24 hours earlier at 2.5×10^6 cells/dish) via calcium phosphate precipitation as previously described (49). Transduction of primary M1-specific T cells was performed 24 hours after DC stimulation by spinning at 300 g for 90 minutes in the presence of 8 μ g/ml polybrene (Sigma-Aldrich). 24 hours after transduction, culture medium containing 1.5 μ g/ml puromycin was used to select for virus-transduced T cells. RNA isolation (Cells-to-CT kit; Invitrogen) and qPCR (TaqMan gene expression assay) for knockdown efficiency and polyfunctionality analysis were performed 6 days after viral transduction. Similar transduction procedures were performed with HIV-specific patients' PBMCs. PBMCs were thawed and rested overnight before activation with soluble anti-CD3 (clone HIT3a, 1 μ g/ml; Biolegend) and anti-CD28 (1 μ g/ml, clone CD28.2; Biolegend) in the presence of

Gag/Nef/Tat/CEF peptide pools and transduced with lentivirus. Anti-PD-1 antibody (10 µg/ml, clone EH12.2H7; Biolegend) was used to block PD-1 signaling. Isotype control antibody (clone MG1-45; Biolegend) was added to control cultures.

[72] HIV-specific T cell sorting and measurement of *SPRY2* expression. HIV Gag antigen specificity was determined by using HLA-A2 restricted PE pentamer (SLYNTVATL; SEQ ID NO:4; Proimmune). CD8⁺ T cells were purified from 7 HLA-A2+HIV patients from the study subjects and stained with HIV-Gag-pentamer (PE) and M1-tetramer (APC). For each donor, more than 1000 antigen-specific T cells were sorted for mRNA extraction and cDNA conversion using the Cells-to-Ct kit (Invitrogen). After preamplification using the TaqMan PreAmp Mastermix Kit (Invitrogen), qPCR was performed using TaqMan gene expression assays, and *SPRY2* expression level were adjusted to *HPRT1* expression. For some donors, unsorted PBMCs were stained with Gag-specific pentamer or M1-specific tetramers, anti-PD-1, and anti-CD8 for an hour. Cells were fixed, permeabilized, and stained with APC-conjugated anti-human *SPRY2* antibody (clone ab60719; Abcam) for an additional 2 hours. Cells were then washed twice before acquisition by flow cytometry.

[73] Statistics. Statistical comparisons between different polyfunctionality pie charts were performed by permutation test set at 10,000 repetitions using SPICE. Other statistical analyses were performed by Prism version 5 (Graph-Pad). For comparison between 2 groups of related samples, the nonparametric Wilcoxon matched-pairs signed rank test was used. The Mann-Whitney *U* test was used to detect the differences between 2 independent groups of samples. A *P* value of less than 0.05 was considered statistically significant.

EXAMPLE 10. Antigen concentration regulates polyfunctionality of human T cells.

[74] Autologous moDCs were pulsed with various concentrations of the immunodominant, HLA-A*0201 restricted influenza peptide M158–66 and used to stimulate CD8⁺ T cells *ex vivo*. On day 14 (D14), after 2 rounds of peptide-pulsed moDC stimulation, cell counts and percentage of M1-specific CD8⁺ T cells were determined. There was a direct correlation between the amount of antigen used, from 10 fM to 10 µM, to pulse DCs and percentage of M1-specific CD8⁺ T cells as well as the overall cell number (**FIGS. 10A-C**). Percentage of M1-specific T cells varied over 40-fold, from approximately 2% to 85%, and a similar impact was seen on total number of M1-specific CD8⁺ T cells.

[75] Antigen concentration also controlled T cell effector function. On D14, T cells were incubated with M1 peptide-pulsed target cells and analyzed for multiple effector functions. Compared with T cells induced by 10 nM peptide-pulsed moDCs, T cells induced by high antigen concentration (10 μ M) had a significantly reduced ability to simultaneously produce multiple cytokines or effector functions (**FIGS. 10D-E**). Only 5.6% of the M1-specific cells induced with 10 μ M peptide-pulsed moDCs produced both IL-2 and CD107, and 15.1% produced both TNF- α and MIP-1 β . In contrast, when T cells were induced with 10 nM moDCs, there were significantly larger percentages of T cells with multiple effector functions. 25.4% of the M1-specific T cells produced both IL-2 and CD107, and approximately 50% produced both TNF- α and MIP-1 β . A low antigen concentration, 10 fM, also induced a lower percentage of dual-function M1-specific T cells when compared with the 10 nM antigen concentration, but a higher percentage of dual function M1-specific T cells compared with the 10 μ M antigen. The effector function most sensitive to antigen concentration was IL-2 production, and functions least sensitive were CD107a and MIP-1 β (**FIG. 10E**). The loss of IL-2 responses during high antigen concentration stimulation is also observed during chronic infections such as HIV (9), where IL-2 production is the most sensitive and first function lost out of other effector functions.

[76] By analyzing overall T cell polyfunctionality, we determined an antigen concentration that induced the maximal amount of polyfunctional T cells. When individual T cell cultures were analyzed for all 5 functions simultaneously, there was a modal distribution for induction of polyfunctional T cells (**FIGS. 10F-G**). 10 nM antigen pulsed moDCs induced the greatest polyfunctional response; 26.4% of all M1-specific T cells had all 5 functions (5+ polyfunctional T cells). This was significantly higher than the response induced by the high antigen concentration, 10 μ M, 4.5% 5+ polyfunctional M1-specific T cells ($P = 0.002$), or by the low antigen concentration, 10 fM, 13.7% 5+ polyfunctional M1-specific T cells ($P = 0.001$). There was no further inhibition of T cell polyfunctionality response to an even higher antigen concentration, 100 μ M. Within the population of 5+ polyfunctional T cells, T cells induced with 10 nM moDCs also produced more IL-2 on a per cell basis (**FIG. 10H**). T cells with the lowest polyfunctionality, induced by 10 μ M moDCs, were dominated by a population of cells capable of producing MIP-1 β and degranulation, but did not produce any cytokines (**FIG. 16**). Upon rechallenge with antigen, T cells induced with 10 nM M1 antigen-pulsed moDCs also exhibited superior cell expansion (**FIG. 17**). In the remainder of this disclosure, we refer to 10 nM as the “optimal antigen concentration” and 10 μ M as the “high

antigen concentration” of peptide used to pulse moDCs. While these studies analyzed the memory M1-specific T cell responses, we also analyzed the impact of antigen concentration on naïve CD8⁺ T cells. When naïve T cells were stimulated with different concentrations of anti-CD3 and anti-CD28, a similar modal distribution for induction of polyfunctional T cells was observed (**FIG. 18**). Optimal concentration of anti-CD3/anti-CD28 resulted in 28% polyfunctional T cells, while the highest concentration of anti-CD3/anti-CD28 led to only 1.8% polyfunctional T cells. Thus, antigen concentration controls T cell polyfunctionality both memory and naïve CD8⁺ T cell populations.

EXAMPLE 11. *Regulation of polyfunctionality is independent of the T cell differentiation markers.*

[77] It is possible that different M1-specific T cell clones are hardwired with different polyfunctionality potential and selectively expanded in response to particular concentrations of antigen. This seemed unlikely as more than 95% of the M1 tetramer-positive cells were positive for V β 17 TCR, independent of the amount of antigen used for stimulation. In addition, it is possible that the T cell differentiation state could also have an impact on their effector functions (19). We characterized T cells stimulated with a range of peptide concentrations using a panel of differentiation markers including CD45RO, CD45RA, CCR7, CD28, and CD27 and found no significant differences (**FIG. 19A**) among the populations. T cells from all stimulation conditions also expressed similar levels of bcl-2 and T-bet (**FIG. 19B**).

EXAMPLE 12. *Molecular signature of T cell polyfunctionality.*

[78] Based on these results, we hypothesized that distinct molecular mechanisms regulate T cell polyfunctionality. To establish the molecular signature of polyfunctionality, antigen-specific T cells were induced by either high or optimal antigen concentration, tetramer sorted on D14, and analyzed for global gene expression. Cut-off point analysis was set at 1.6-fold change and an adjusted *P* value of less than 0.05. optimal antigen concentration-induced T cells, and 302 genes were down-regulated (**FIG. 11A**). Functional grouping performed by the Database for Annotation, Visualization, and Integrated Discovery (DAVID) revealed that most differentially regulated genes are central to lymphocyte function, including inhibitory receptor expression, lymphocyte activation, chemotaxis, cytotoxicity, and antigen presentation (**Supplementary Table 1**). A full list of differentially expressed genes can be found in **Supplementary Table 2**. Compared with high antigen

concentration–induced T cells, highly polyfunctional T cells induced by the optimal antigen concentration expressed less inhibitory receptors at the mRNA level, including PD-1, 2B4 (CD244), and KLRG1. Microarray data showed that transcription factors that promote CD8⁺ T cell memory formation, such as *EOMES* (20) and *TCF7* (21), were higher in highly polyfunctional T cells, and differences were confirmed by flow cytometry or quantitative PCR (qPCR) (**FIGS. 11B-C**). We also found differential expression of multiple chemokines and chemokine receptors, which have also been shown to regulate T cell differentiation and memory formation (22, 23), and a large group of differentially expressed genes were involved in signal transduction.

EXAMPLE 13. *High antigen concentration–induced T cells are enriched with the molecular signature of T cell exhaustion.*

[79] GSEA has been used to compare genomic signatures between microarray experiments in order to integrate different phenotypic cellular states (24). GSEA showed that highly polyfunctional T cells were significantly enriched with the genetic signature for memory T cells (ref. 25 and **FIG. 11D**). In contrast, there was a significant enrichment of the exhausted gene signature (25) among high antigen concentration–induced T cells, indicating that these T cells share aspects of global gene expression patterns with T cells that are exhausted due to chronic LCMV infection (**FIG. 11D**). Similar enrichment was found while using the differentially expressed gene list from HIV progressors versus controllers (26). Interestingly, while it has been established that the PD-1/PD-L1 pathway is involved in T cell exhaustion during chronic viral infections (27), GSEA analysis of the PD-1 ligation signature (26) showed no significant enrichment.

EXAMPLE 14. *Inhibitory receptor signaling is not required for high antigen concentration–induced polyfunctionality inhibition.*

[80] To specifically study the role of inhibitory receptor signaling in the generation of polyfunctional T cells, we analyzed the inhibitory receptor expression on T cells induced by different antigen concentrations. Significantly higher levels of multiple inhibitory molecules such as PD-1, CTLA-4, and TIM-3 were seen in M1-specific T cells induced by high versus optimal concentration of antigen (**FIG. 12A**). However, no difference was seen in expression of another inhibitory receptor, BTLA. We took several approaches to analyzing the potential role of signaling through inhibitory receptors, such as PD-1. Expression of basic leucine transcription factor, ATF-like (BATF), an important PD-1 downstream signaling

molecule (26), was not upregulated by qPCR, in high antigen concentration–induced T cells (**FIG. 12B**). We further analyzed the potential role of PD-1 during high antigen concentration stimulation using anti-PD-1 antibody to block PD-1/PD-L1 interaction (10 µg/ml, clone EH12.2H7). Nevertheless, no increase in polyfunctionality was seen in high antigen concentration–induced T cells treated with anti-PD-1 (**FIG. 12C**). In addition to PD-1, there are other inhibitory ligands present on DCs that could potentially modulate polyfunctionality. To analyze the impact of antigen concentration on polyfunctionality in the absence of potential inhibitory molecules, we used artificial APCs (aAPCs), which have no inhibitory receptor ligands. This is a reductionist system in which aAPCs are made by conjugating HLA-A2–Ig-M1 complex (signal 1) and anti-CD28 (signal 2) onto particles (28, 29). Higher concentrations of aAPCs with signal 1 plus signal 2 induced significantly more antigen-specific T cell proliferation (**FIGS. 12D-E**), but lower T cell polyfunctionality (**FIG. 12F**). Similar to moDC stimulation, there was an optimal antigen concentration for aAPC stimulation. Therefore, while signaling through PD-1 and other inhibitory receptors is widely involved in T cell exhaustion, polyfunctionality regulation due to antigen concentration requires only signal 1 and signal 2.

EXAMPLE 15. *MAPK/ERK pathway controls T cell polyfunctionality in response to antigen concentration.*

[81] To further investigate the molecular mechanism regulating polyfunctionality, we performed GO-BP-FAT analysis on the microarray result and found that the MAPK/ERK pathway is altered in T cells with different levels of polyfunctionality (**Supplementary Table 3**). The MAPK/ERK pathway is an important signaling pathway that critically regulates a variety of physiological processes, such as cell growth, differentiation, and survival. This pathway also plays important roles in many aspects of lymphocyte biology, such as thymocyte selection, CD4⁺ T cell differentiation, and T cell activation (30). However, the role of the MAPK/ERK pathway in T cell polyfunctionality and exhaustion has not been reported. Differences were seen in the level of phospho-ERK (pERK) upregulation in T cells induced by either high or optimal antigen concentration. 67% of the optimal antigen concentration–induced T cells upregulated pERK in response to stimulation, while only 33% of the poorly polyfunctional T cells showed increased levels of pERK (**FIG. 13A**). Upregulation of pERK was shown to be specific, as treatment of cells with U0126, a MEK1/2 specific inhibitor, inhibited ERK phosphorylation in a concentration-dependent fashion, with complete inhibition of pERK seen with 100 µM U0126 (**FIG. 14B**). If the MAPK/ERK

pathway differentially controls effector functions involved in polyfunctionality, such as cytokine production, one should be able to convert 5+ polyfunctional antigen-specific T cells to low polyfunctional T cells by inhibiting ERK phosphorylation. In the presence of U0126, there was a dose-dependent inhibition of cytokine production (IL-2, TNF, and IFN- γ), with little effect on CD107 and MIP-1 β expression in optimal antigen concentration-induced T cells. There was an approximately 10-fold reduction in the amount of IL-2 and TNF- α made by M1-specific CD8 $^{+}$ T cells (**FIGS. 11C-D**). However, CD8 $^{+}$ T cell-mediated lysis of target cells was largely insensitive to U0126 treatment (**FIG. 13E**). Overall, the data indicate that cytokine secretion and polyfunctionality but not lytic ability or MIP-1 β expression is dependent on the MAPK/ERK pathway. Thus, the differential consequences of optimal and high antigen concentrations could be replicated by biochemical inhibition of the MAPK/ERK pathway.

EXAMPLE 16. Upregulation of *SPRY2* in high antigen concentration-induced T cells inhibits polyfunctionality.

[82] One gene identified in the microarray gene list (**Supplementary Table 1**) known to affect the MAPK/ERK pathway is *SPRY2*. Sprouty proteins are a well-conserved family known to mediate the negative feedback regulation of the MAPK pathway (31, 32). Sprouty proteins bind to Grb2 and other components of the MAPK pathway, preventing the upregulation of pERK (33, 34). qPCR analysis confirmed that *SPRY2* is upregulated in high antigen concentration-induced CD8 $^{+}$ T cells (**FIG. 14A**) and upregulation of *SPRY2* is downstream of ERK activation, as it is inhibited by U0126 treatment added during high antigen concentration stimulation (**FIG. 20**). To determine whether *SPRY2* mediates suppression of polyfunctionality in high antigen concentration-induced T cells, we engineered lentiviral particles containing shRNA-targeting *SPRY2* in order to inhibit its expression. *SPRY2* knockdown virus efficiently inhibited mRNA expression of *SPRY2*, by approximately 80%, while control non-target (NT) virus had no effect (**FIG. 14B**). *SPRY2* knockdown also led to a significant decrease in *SPRY2* protein levels as determined by flow cytometry (**FIG. 14B**). The MFI of *SPRY2* in the *SPRY2*knockdown cells decreased from 1860 to 910. Upon antigen stimulation, the *SPRY2* knockdown T cells produced more cytokines than the control lentivirus-infected T cells (**FIG. 14C**), and overall, the percentage of 5+ polyfunctional T cells increased from approximately 21% to 37% (**FIG. 14D**). Thus, high antigen concentration stimulation leads to lower CD8 $^{+}$ T cell polyfunctionality through upregulation of *SPRY2*, which inhibits the MAPK/ERK pathway.

EXAMPLE 17. *SPRY2* controls HIV-specific T cell polyfunctionality.

[83] As compared with acute infection such as influenza, HIV-specific CTL responses in infected patients are known to have significantly lower levels of polyfunctionality, and CTL dysfunction is not restored by antiretroviral therapy (9, 35). Recent studies indicate that inhibition of the PD-1 pathway partially reverses low polyfunctionality associated with chronic viral infection (13). However, the effects of anti-PD-1 treatment were modest (14, 15) and indicated that additional mechanisms control HIV-specific T cell polyfunctionality. We hypothesized that HIV-specific T cells also upregulate *SPRY2* and that upregulation of *SPRY2* is responsible for the low levels of T cell polyfunctionality seen in HIV-specific responses. PBMCs from 7 HLA-A2-positive HIV-infected patients were sorted on the basis of pentamer/tetramer staining into either HIV Gag- or influenzaM1-specific CTL (**FIG. 15A**). Within CD8⁺ T cells, the average percentage of Gag-specific T cells was 0.45% as compared with 0.04% of M1-specific T cells ($P = 0.03$). qRT-PCR on these populations showed that *SPRY2* was upregulated in the Gag-specific T cells compared with both the M1-specific T cells and nonspecific CD8⁺ T cells from the same donors. Flow cytometry staining for *SPRY2* showed that Gag-specific T cells express higher levels of *SPRY2* protein and PD-1 (**FIG. 15B**). Consistent with previous findings (9), these cells are less polyfunctional when analyzed directly *ex vivo* (**FIG. 21**). We next studied the effect of *SPRY2* knockdown on HIV Gag-, Nef-, and Tat-specific CTL responses (**FIG. 15C–F**). Inhibition of *SPRY2* enhanced all the HIV-specific polyfunctional responses independently of PD-1 blockade. In addition, inhibition of *SPRY2* in combination with anti-PD-1 treatment further augmented polyfunctional responses (**FIG. 15C–F**). By combining anti-PD-1 with *SPRY2* inhibition, HIV Gag-specific polyfunctionality improved to levels seen in the CEF-specific (CMV, EBV, and influenza) responses. In contrast, neither *SPRY2* inhibition nor PD-1 blockade had a significant effect on CEF-specific responses from the same donors (**FIG. 15F**). These findings indicate that *SPRY2* upregulation inhibited HIV-specific T cell polyfunctionality independently of the PD-1 pathway.

Discussion

[84] Polyfunctionality has emerged as a highly significant predictor of protective immunity (1, 2, 7, 9, 18). In this report, we generated antigen concentration-dependent influenza-specific CD8⁺ T cells with differing levels of polyfunctionality. An optimal concentration of antigen, resulting in highly polyfunctional T cells, was determined, and microarray analysis showed that these T cells were enriched for a memory T cell signature. In

contrast, high antigen concentration–induced T cells that were significantly less polyfunctional were enriched for a T cell exhaustion signature. These poorly polyfunctional T cells had high levels of inhibitory receptor expression, but inhibitory receptor signaling did not regulate polyfunctionality in this system. By utilizing the aAPC system, we demonstrated that high antigen concentration alone could regulate polyfunctionality. Through these findings, we identified the MAPK/ERK pathway and *SPRY2* as regulators of polyfunctionality in high antigen concentration stimulation and validated the importance of *SPRY2* in chronic HIV infection.

[85] The molecular profile of highly polyfunctional T cells is enriched with memory signature genes and is consistent with the idea that the polyfunctional state is associated with memory T cell development (1, 19, 36). Two key genes identified as part of the polyfunctional signature were *EOMES* and *TCF7*, both known to be indispensable for optimal CD8⁺ T cell memory formation (20, 37). The mechanisms governing their expression levels are not entirely clear, but here we identified antigen concentration as an important factor. The finding that optimal antigen concentration induced higher levels of *TCF7* and *EOMES* could identify the molecular mechanism linking optimal antigen concentration to superior T cell memory formation.

[86] High antigen concentration induced the most robust proliferation, but the lowest levels of T cell polyfunctionality. The dominant subpopulation of high antigen concentration–induced T cells did not produce any cytokines, but these T cells efficiently mediated target cell recognition and killing. Thus, high antigen concentration–induced T cells were not anergized. These T cells, which exhibit poor cytokine production, low proliferative potential, and high levels of KLRG1, may represent short-lived effector T cells driven toward terminal differentiation during antigenic stimulation, albeit they did not yet exhibit the CD28⁻ and CD27⁻ phenotype (**FIG. 19**).

[87] Chronic viral infection is also associated with high antigen load, T cell exhaustion, and the loss of polyfunctionality. The loss of each individual function in our system follows the hierarchical order for loss of functions observed in HIV and LCMV infections (8). In brief, IL-2, a homeostatic cytokine that promotes T cell proliferation, is lost first. Subsequently, effector cytokines such as TNF- α and IFN- γ secretion are impaired. In contrast, upregulation of CD107a and MIP-1 β are least affected (9). Enrichment of the T cell exhaustion signature in the high antigen concentration–induced T cells indicates that these T cells share certain biological pathways with exhausted T cells seen in HIV (26) and chronic

LCMV (25) infection. Thus, high antigen concentration–induced T cells may represent cells “on their way” to becoming completely exhausted.

[88] Inhibitory receptor upregulation is a key feature of T cell exhaustion (10) and often characterized by active suppression mediated through the PD-1/PD-L1 pathway (13, 14). Although the poorly polyfunctional T cells expressed more inhibitory receptors, the poorly polyfunctional phenotype was independent of inhibitory receptor signaling. This is evident by the fact that PD-1/PD-L1 blockade did not attenuate high antigen concentration–induced loss of polyfunctionality and that the effect of high antigen concentration was seen even when using a signal 1/2 only–aAPC system to induce poorly polyfunctional T cells. Additional evidence comes from the lack of enrichment of the PD-1 molecular signature, such as upregulation of BATF, in the high antigen concentration–induced T cells. Overall, our data show that TCR signaling strength alone determines T cell polyfunctionality.

[89] Our study identified *SPRY2* as a regulator of T cell polyfunctionality and showed that *SPRY2* expression level is dependent on antigen-concentration. Sprouty proteins were originally identified in *Drosophila* as regulators for embryonic development, and mutations in sprouty proteins were found in various human cancers (31). All sprouty proteins were found to be negative regulators of growth factor signaling, especially the MAPK/ERK pathway (32, 33).

[90] A potential role of sprouty proteins has been seen in T cell activation where both *SPRY1* and *SPRY2* inhibit T cell activation and IL-2 production (34, 38). *SPRY1*-deficient mice have been shown to have enhanced antitumor T cell responses (39). Interestingly, a recent microarray analysis indicated that *SPRY2* is upregulated in CD8+CXCR1–CD27–CD28– T cells capable of producing IL-2 (40). However, the physiological role of *SPRY2* was not validated in these studies. Given the importance of the MAPK pathway in immune system signaling, it is likely that sprouty proteins could also regulate the outcome of diverse immune responses. During immune response against infection, upregulation of sprouty proteins might be beneficial to the host as a strategy for preventing immune system overactivation and subsequent immunopathology. On the other hand, modulation of *SPRY2* expression could be a potential way to enhance T cell polyfunctionality and immunological memory during chronic infections.

[91] Authors of several previous reports have tried to improve HIV or HCV virus–specific polyfunctional responses by blocking inhibitory receptor interaction with the

receptors' ligands, such as PD-1/PD-L1 (13–15, 41). To date, the improvement in T cell function has been modest and probably is limited only to less terminally differentiated cells (42). Here, we show that inhibition of *SPRY2* expression was associated with improvement of HIV-specific polyfunctional responses above and beyond the effects of PD-1 blockade. As a result, *SPRY2* could be a novel therapeutic target for reversing T cell exhaustion to be used in combination with inhibitory receptor blockade. The finding that CEF-specific T cells, which remain highly polyfunctional in HIV patients, did not respond to *SPRY2* inhibition indicates that the expression level of *SPRY2* could be related to different T cell outcomes of viral infections and additional studies on the regulatory mechanisms of its expression are warranted.

[92] Our study mechanistically reveals the need to choose optimal antigen doses for immunization to achieve effective immunological memory and protection. While it is hard to extrapolate from the doses of antigen used in our in vitro studies to specific vaccine doses, simply increasing the dose of antigen could result in generation of poorly polyfunctional T cells with less memory potential and might not be an appropriate approach for at-risk populations who exhibit inferior responses to standard vaccinations. Thus, the human T cell polyfunctionality signature and the molecular mechanisms involving *SPRY2* and the MAPK/ERK pathway, as identified in the current study, can have important clinical applications such as identifying desirable immune responses in response to immunization and infection.

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CLAIMS

1. A method of inducing T cell polyfunctionality, comprising inhibiting *SPRY2* expression.
2. The method of claim 1, wherein *SPRY2* expression is inhibited by an agent selected from the group consisting of a Spry2-targeted ribozyme, a Spry2-targeted antisense oligonucleotide, a Spry2-targeted siRNA, an aptamer, a protein, a peptide, a cyclic peptide, a peptidomimetics, a small molecule, and a polypeptide fragment or mutant form of a Spry2 protein which functions as a competitive inhibitor of a Spry2-interacting polypeptide.
3. A method of inducing T cell polyfunctionality, comprising activating the Wnt/ β -catenin signaling pathway.
4. The method of claim 2, wherein the Wnt/ β -catenin signaling pathway is activated by inhibiting serine-threonine kinase glycogen synthase kinase-3 β .
5. A method of treating chronic infection or cancer, comprising administering to a patient in need thereof a therapeutic amount of an agent that inhibits *SPRY2* expression.
6. A method of treating chronic infection or cancer, comprising administering to a patient in need thereof a therapeutic amount of an agent that activates the Wnt/ β -catenin signaling pathway.
7. Use of an agent that inhibits *SPRY2* expression in the manufacture of a medicament for inducing T cell polyfunctionality.
8. Use of an agent that activates the Wnt/ β -catenin signaling pathway in the manufacture of a medicament for inducing T cell polyfunctionality.
9. Use of an agent that inhibits *SPRY2* expression to treat chronic infection or cancer.
10. Use of an agent that activates the Wnt/ β -catenin signaling pathway to treat chronic infection or cancer.

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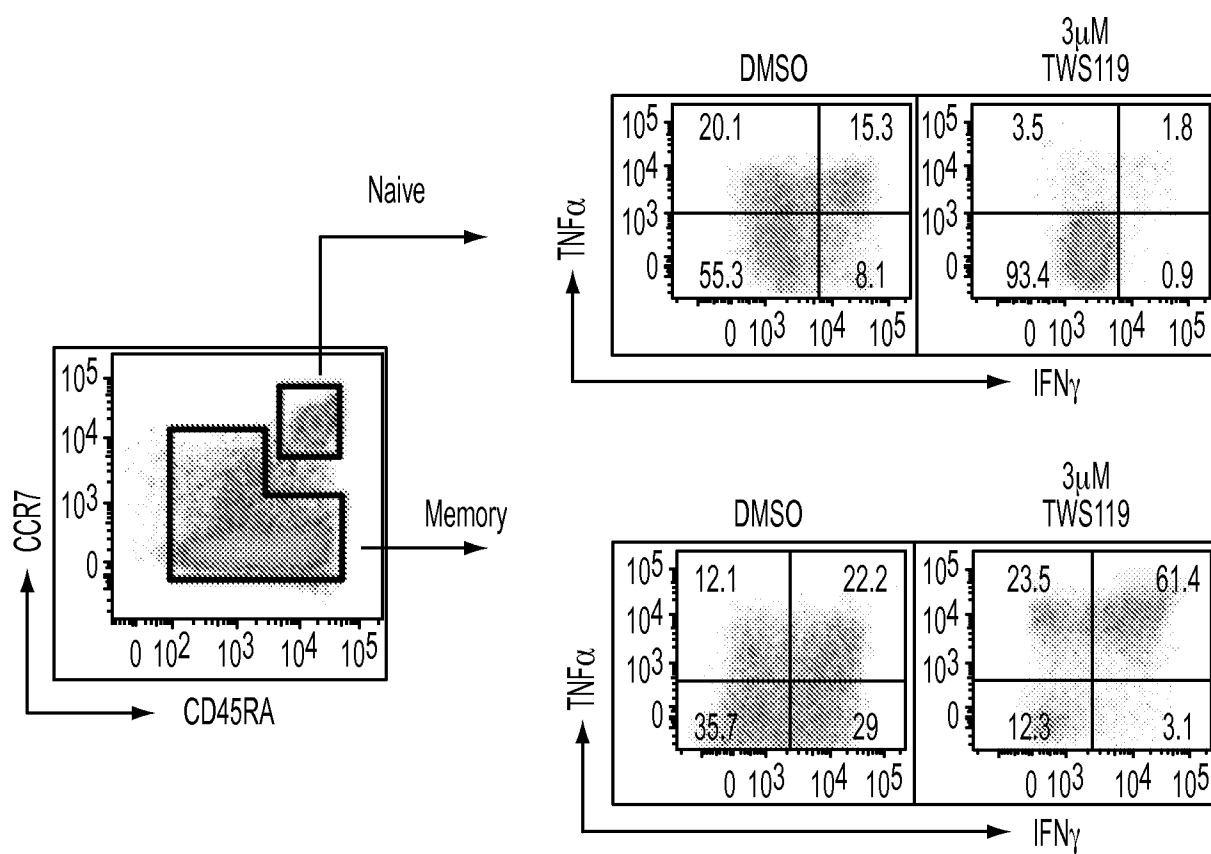


FIG. 1A

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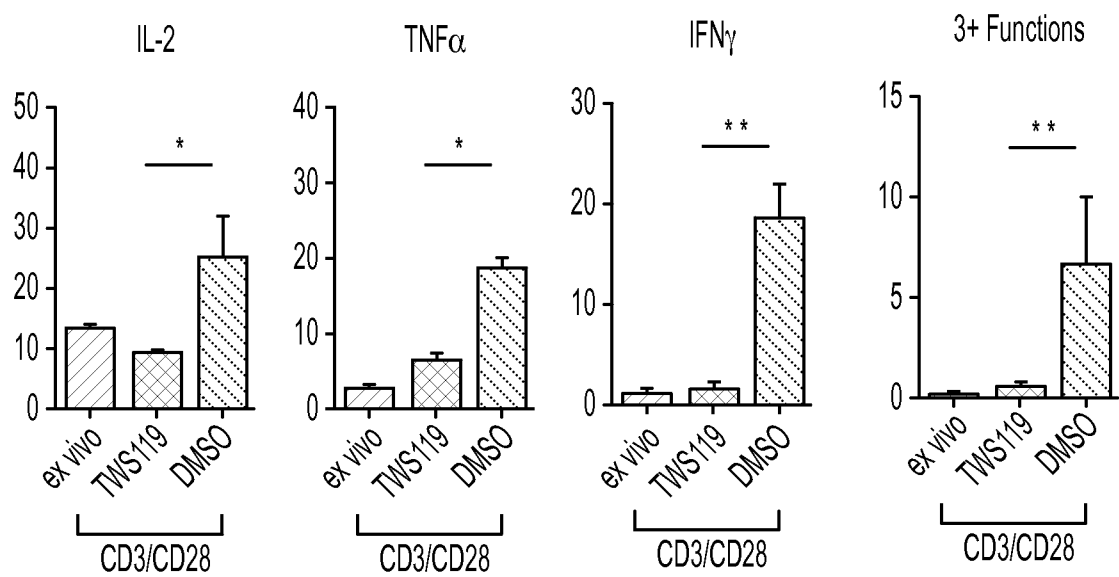


FIG. 1B

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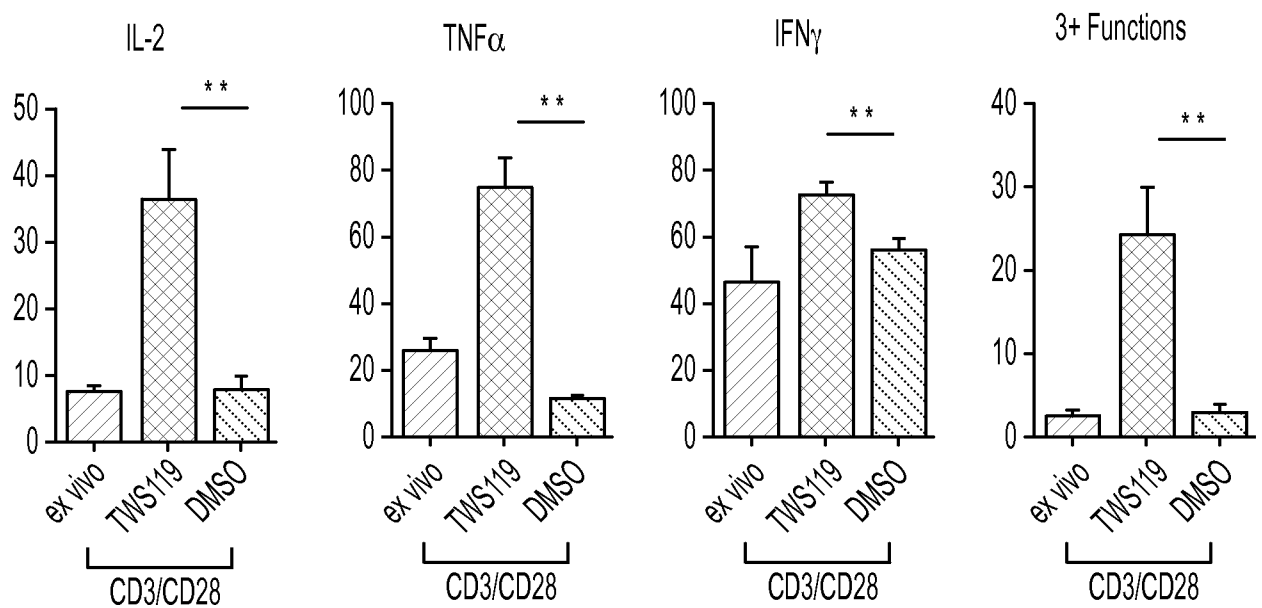


FIG. 1C

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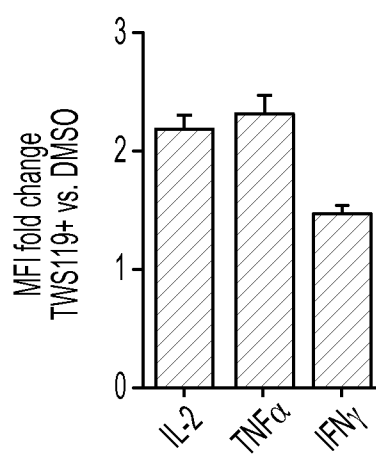


FIG. 1D

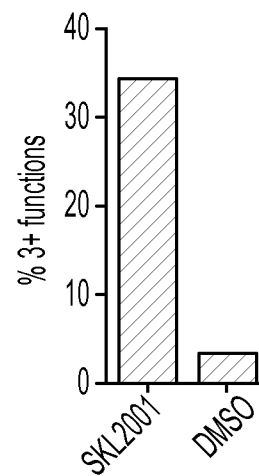


FIG. 1E

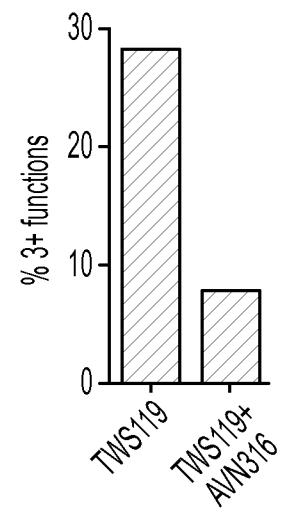


FIG. 1F

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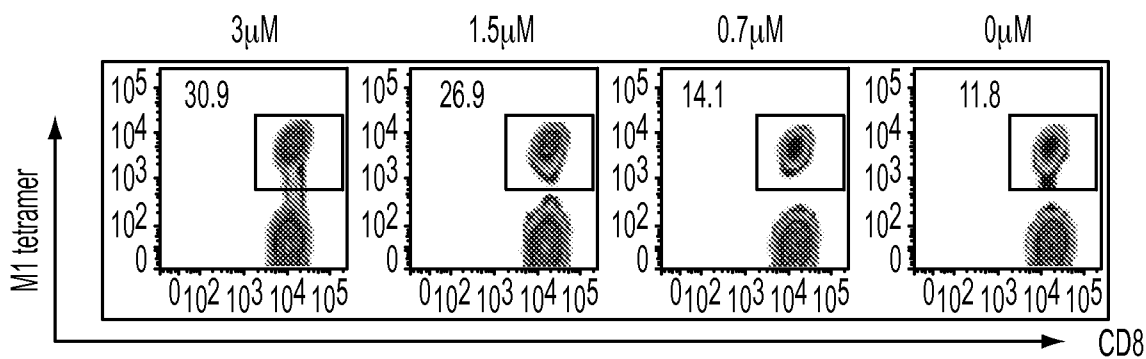


FIG. 2A

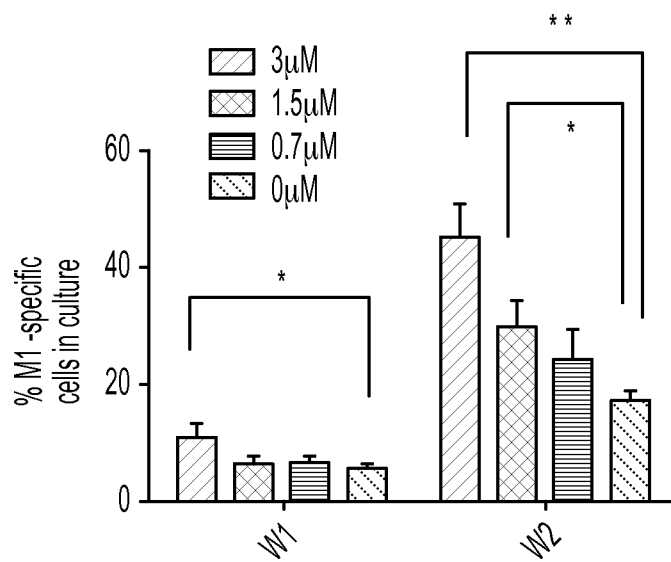


FIG. 2B

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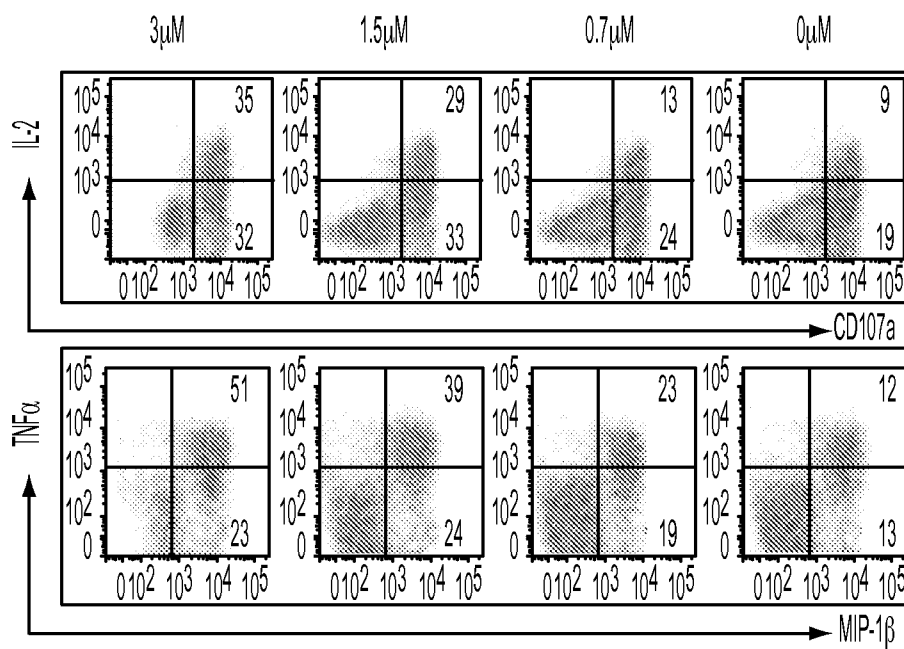


FIG. 2C

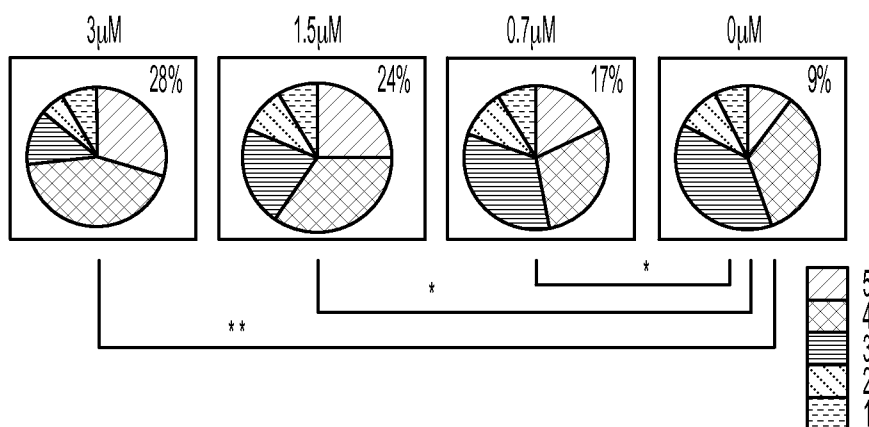


FIG. 2D

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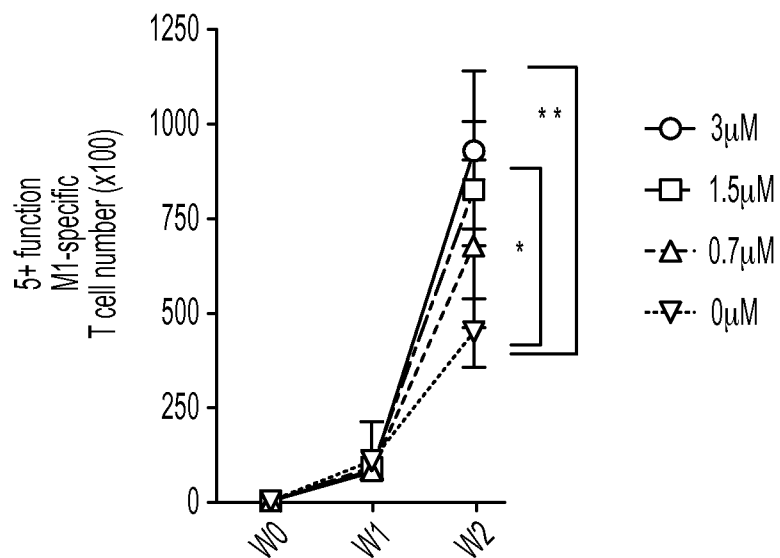


FIG. 2E

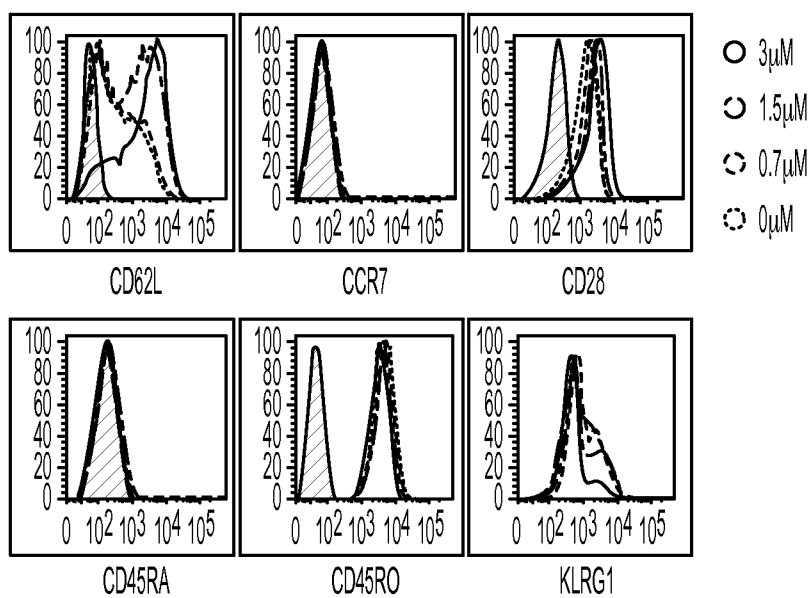


FIG. 2F

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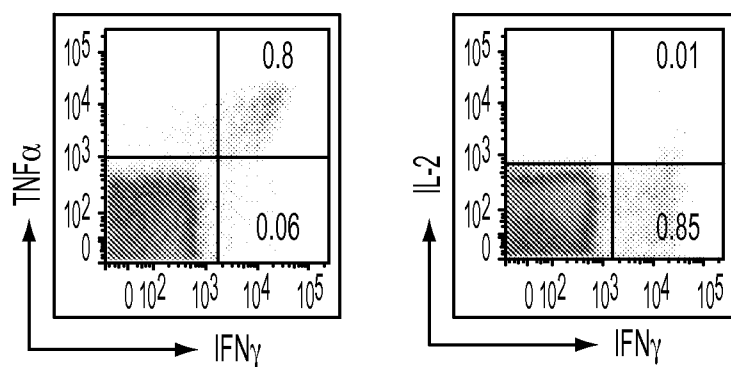


FIG. 3A

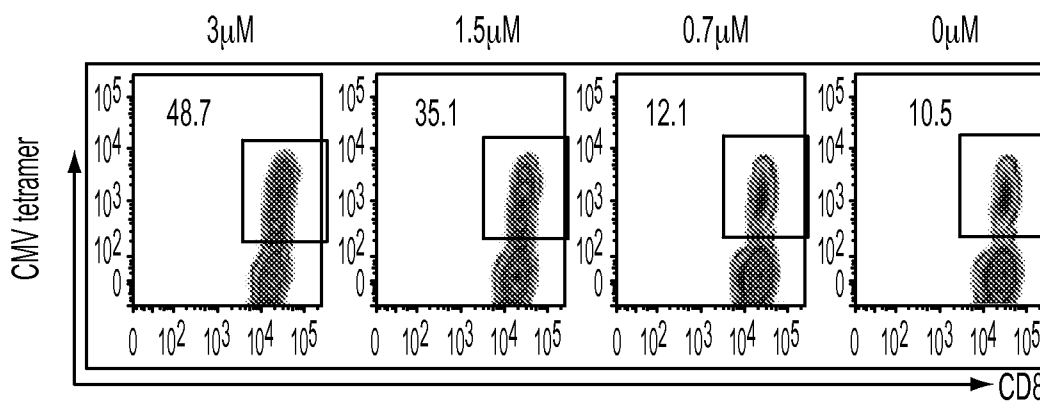


FIG. 3B

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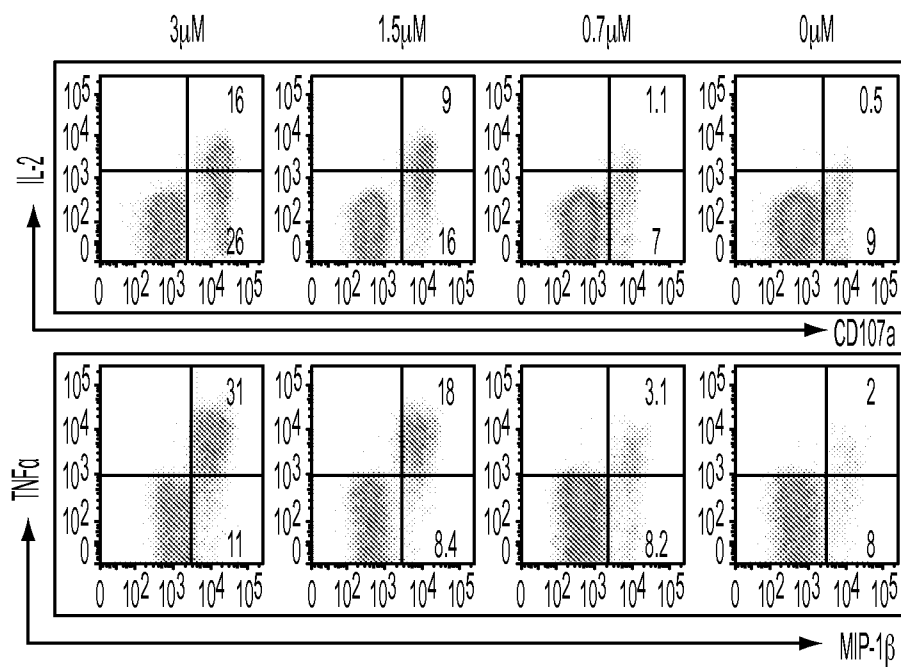


FIG. 3C

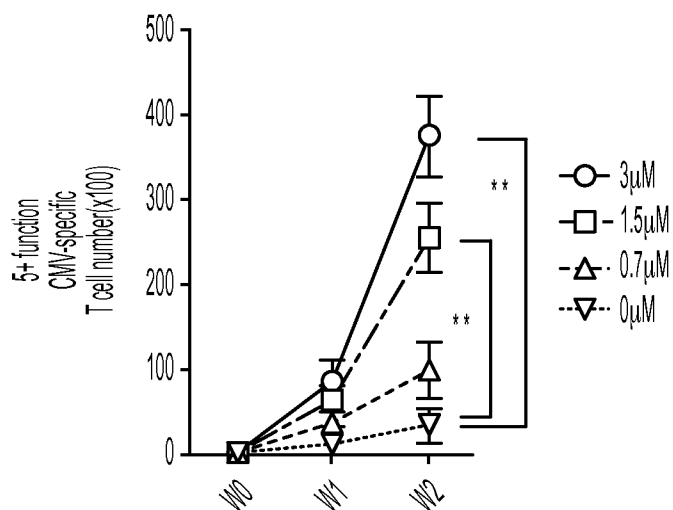


FIG. 3D

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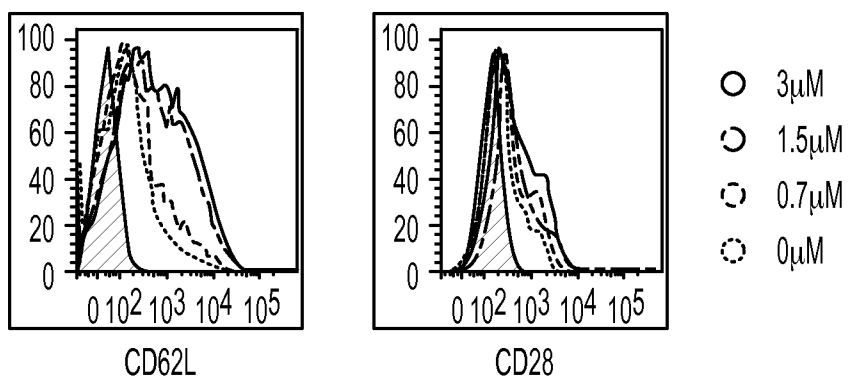


FIG. 3E

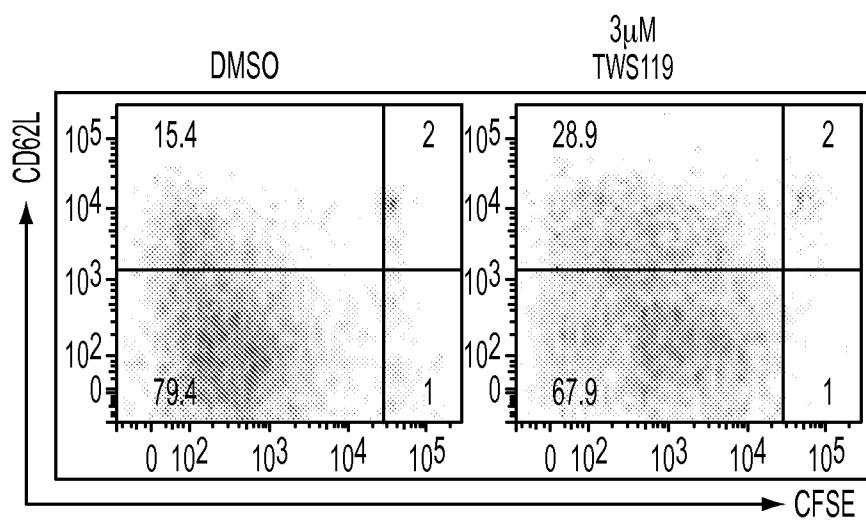


FIG. 4A

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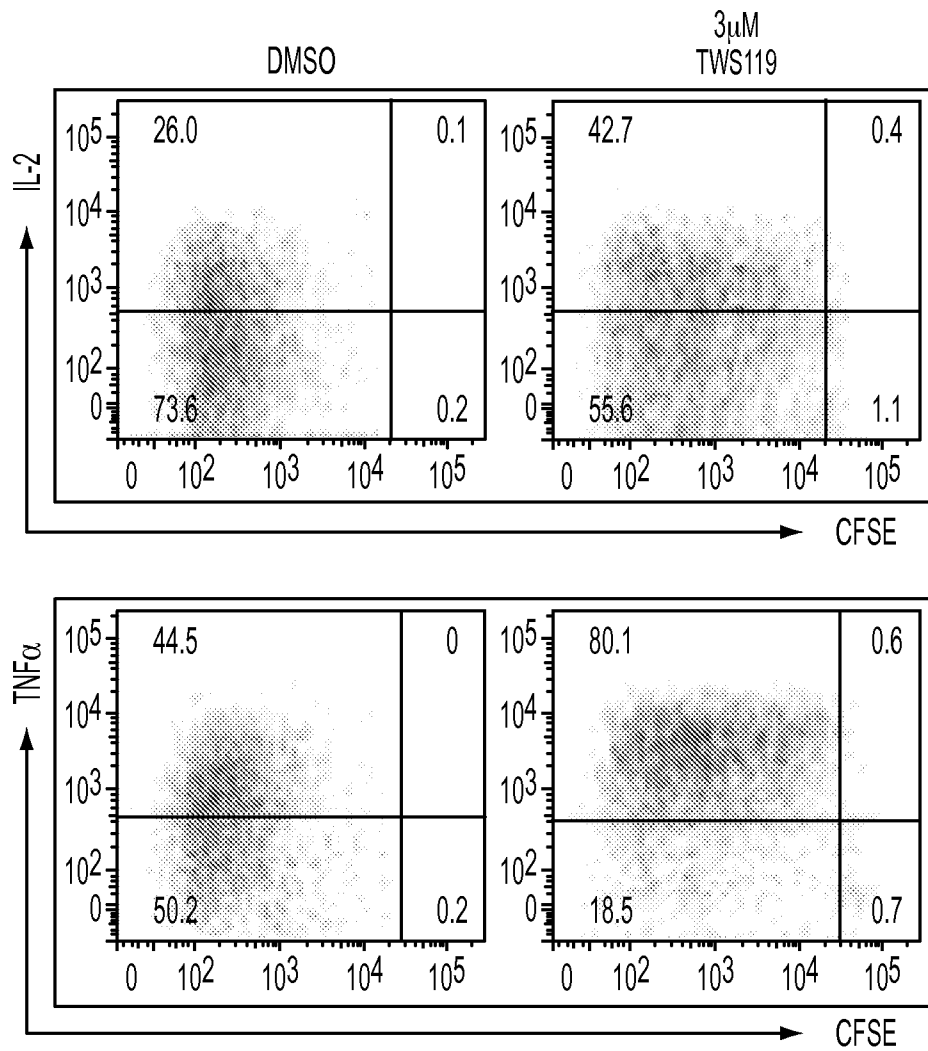


FIG. 4B

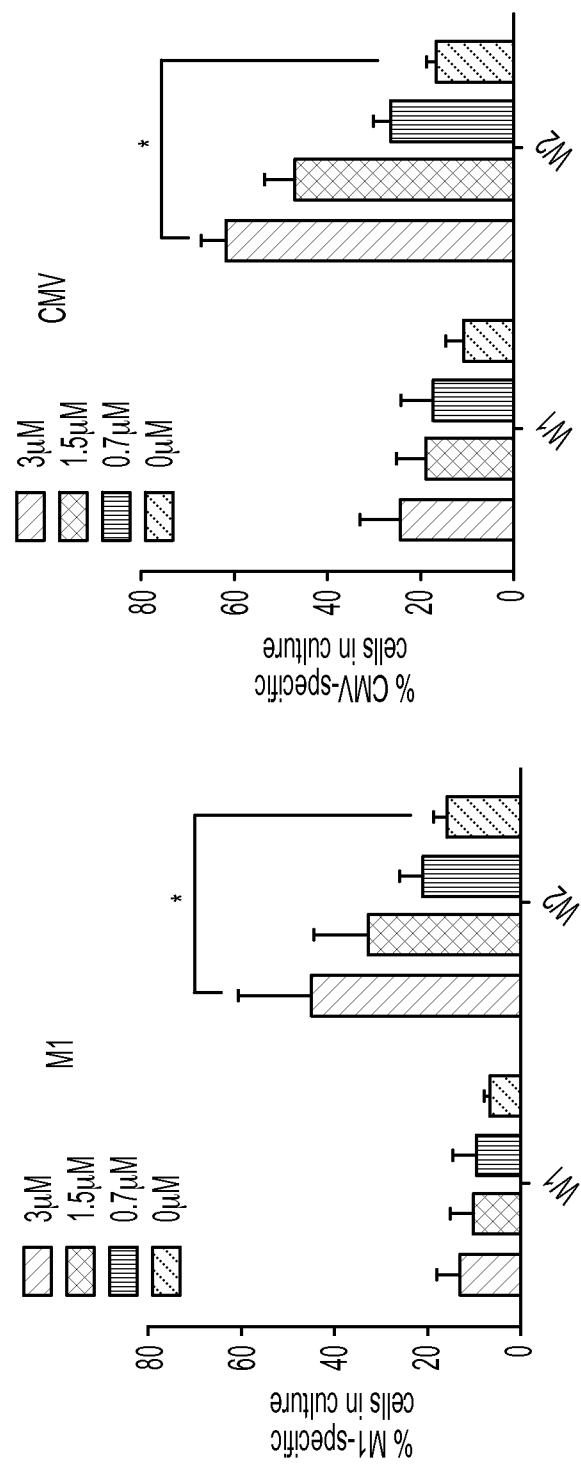


FIG. 4C

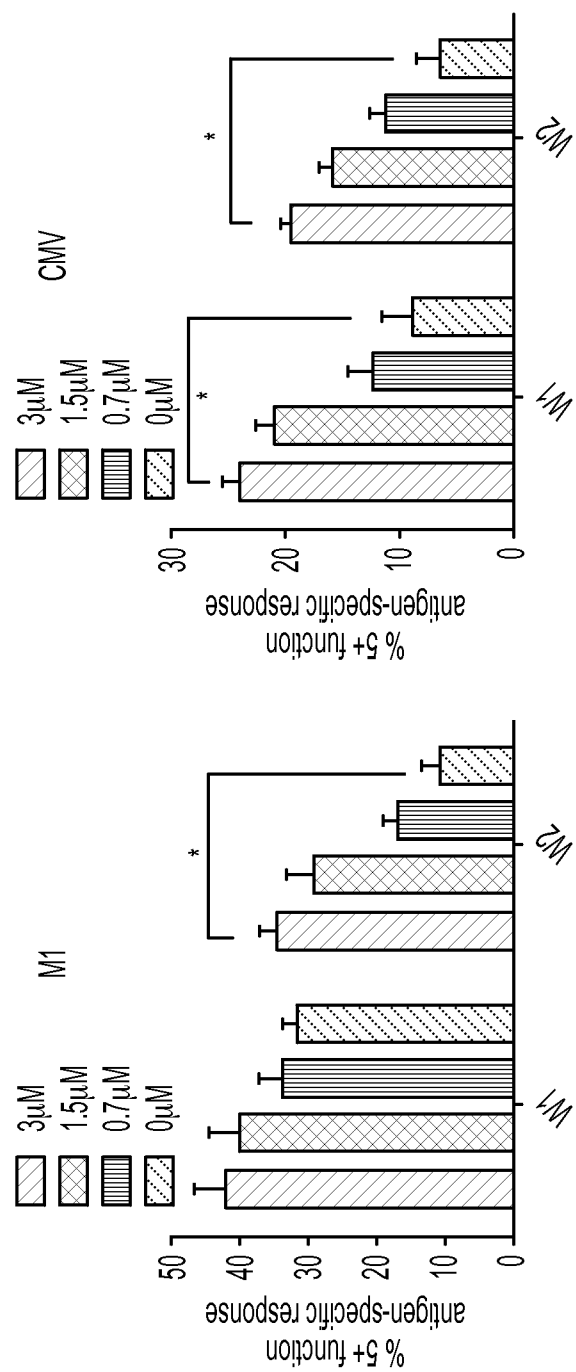


FIG. 4D

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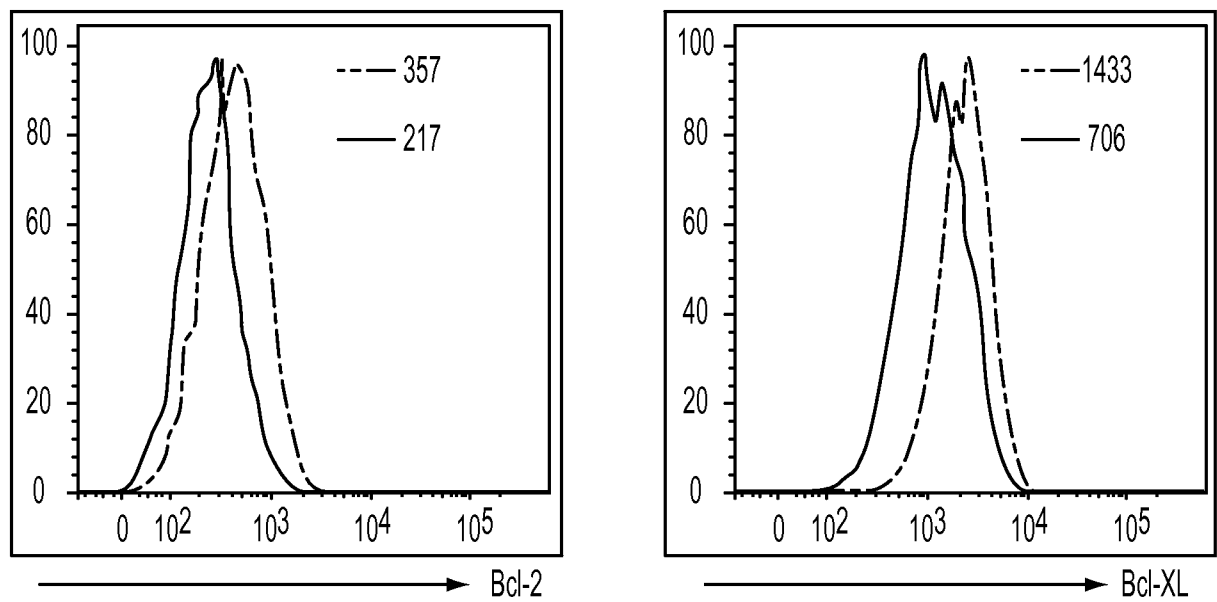


FIG. 5A

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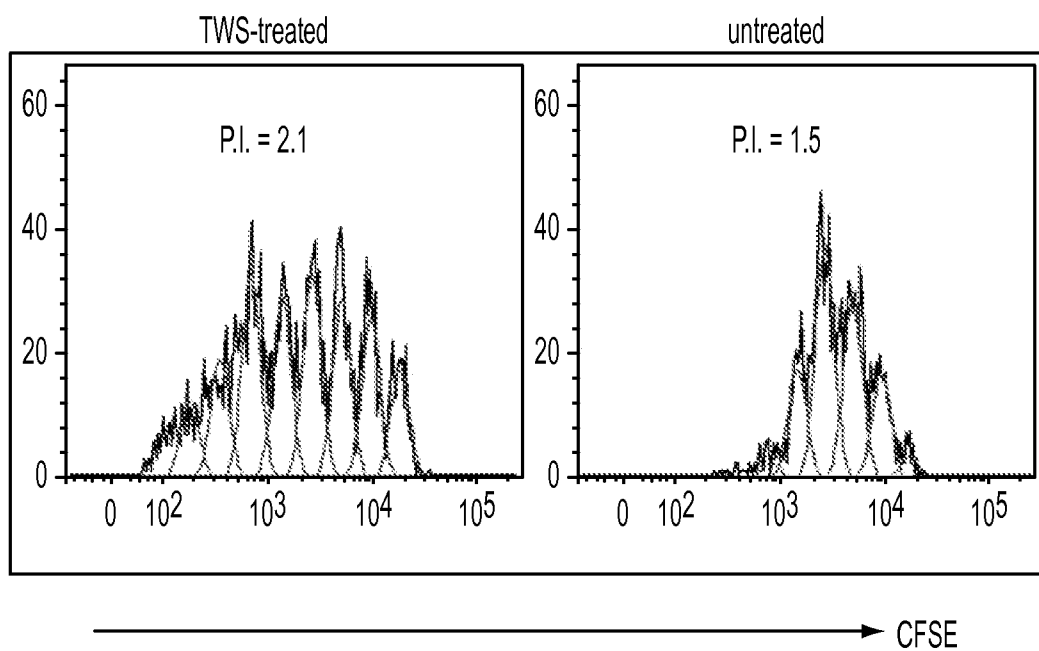


FIG. 5B

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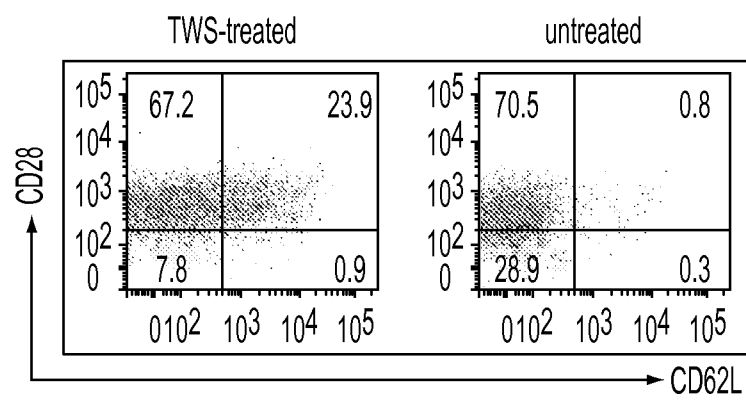


FIG. 5C

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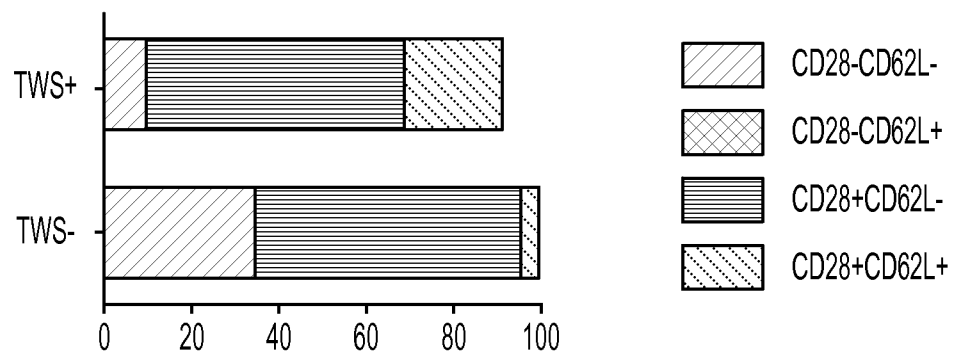


FIG. 5D

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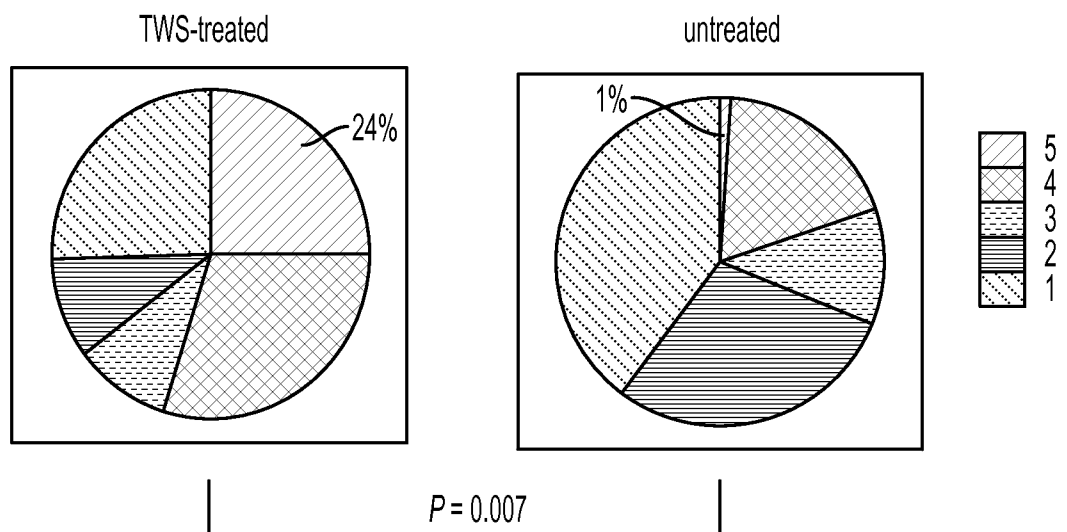


FIG. 5E

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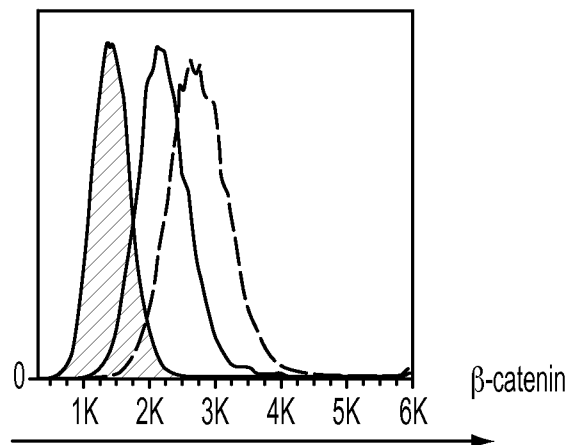


FIG. 6A

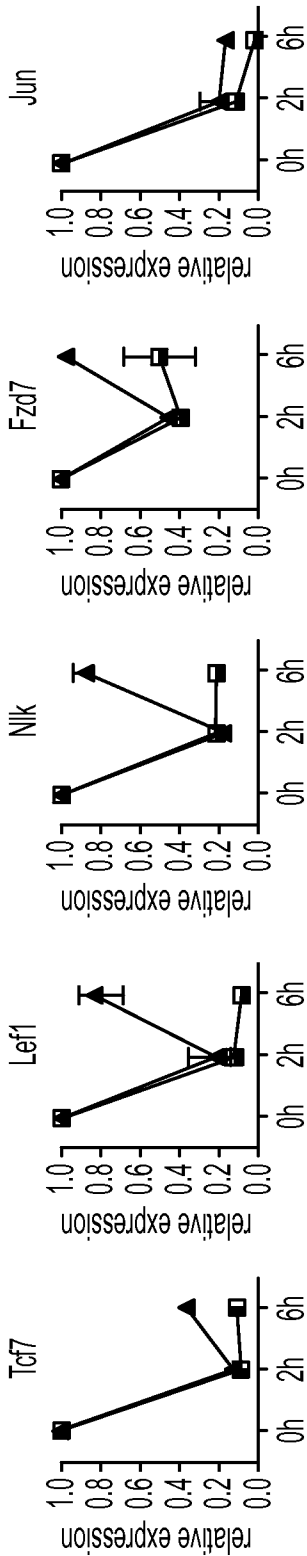


FIG. 6B

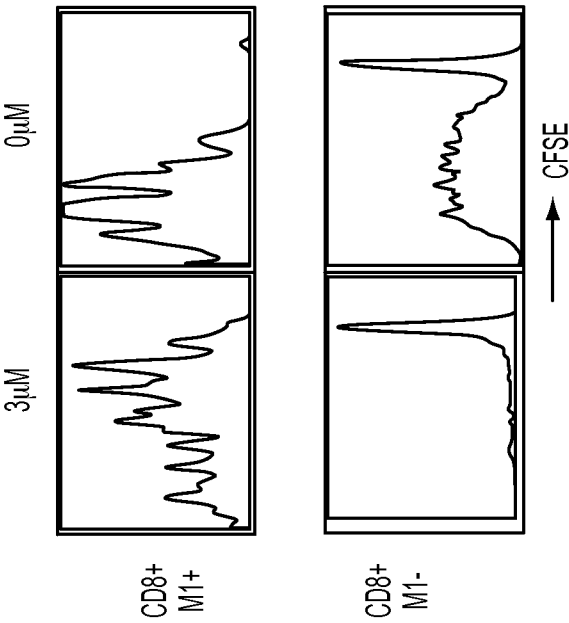


FIG. 7C

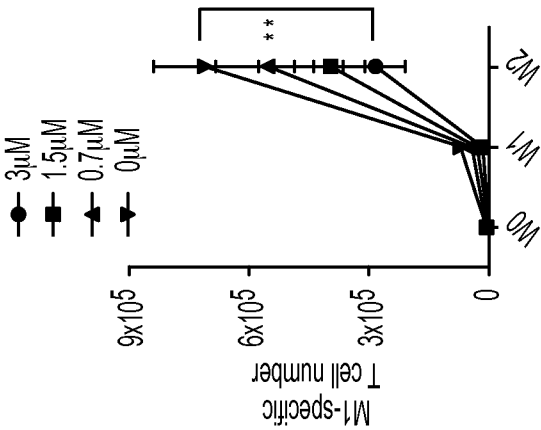


FIG. 7B

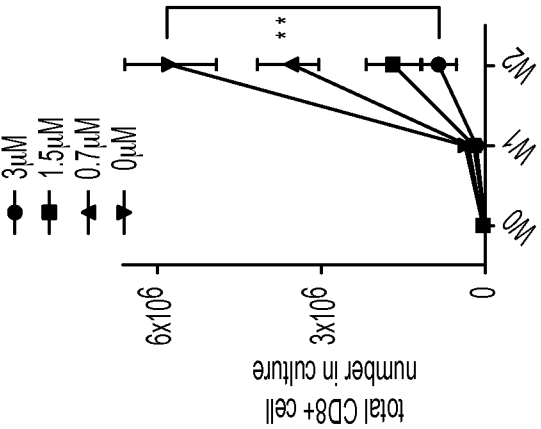


FIG. 7A

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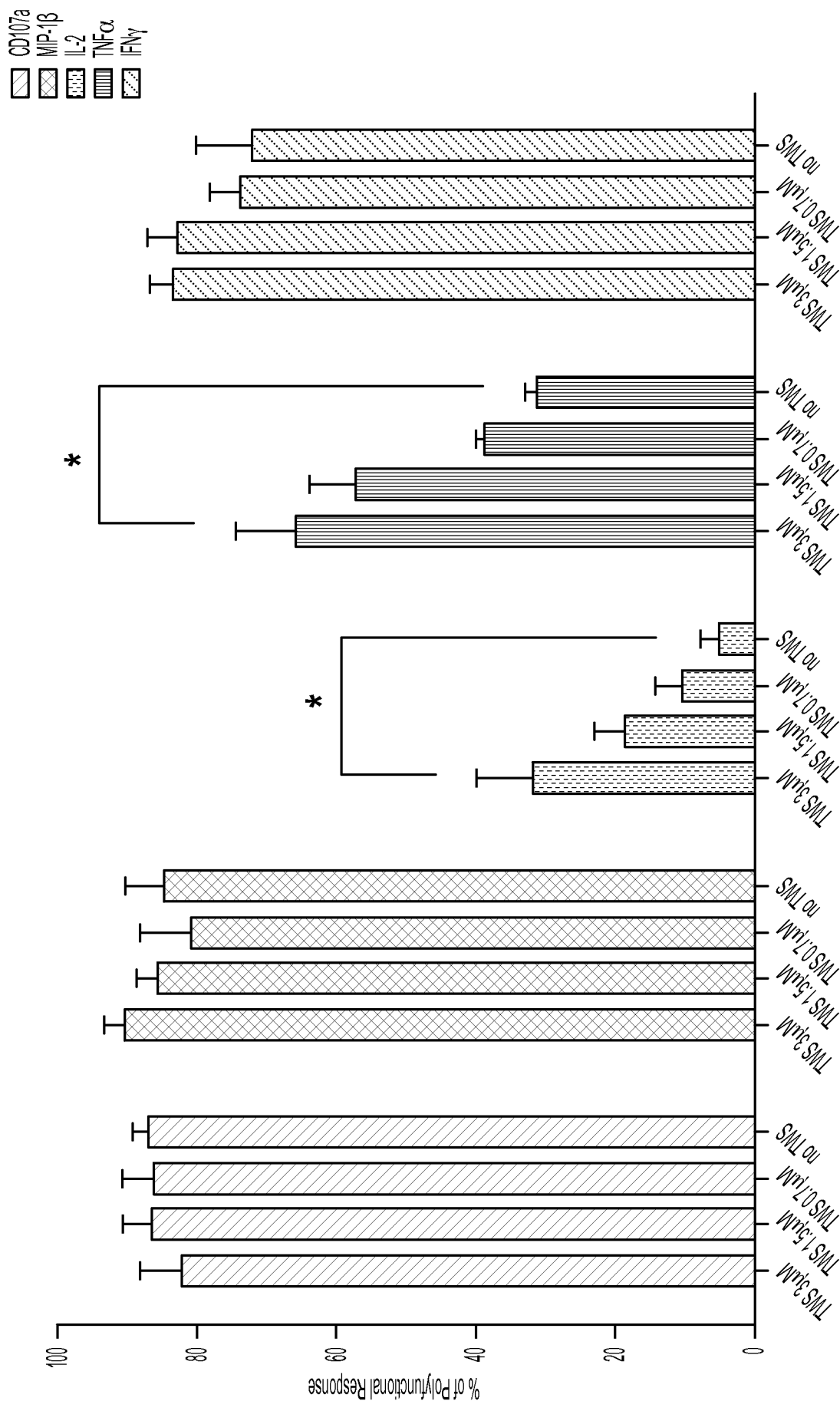


FIG. 8

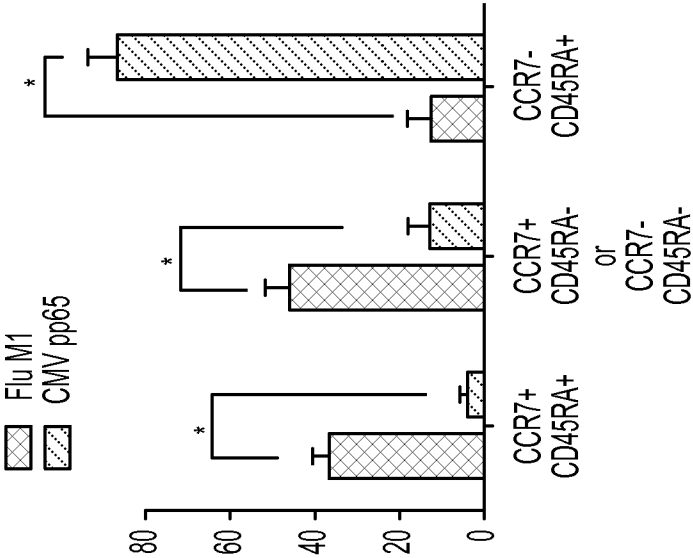


FIG. 9B

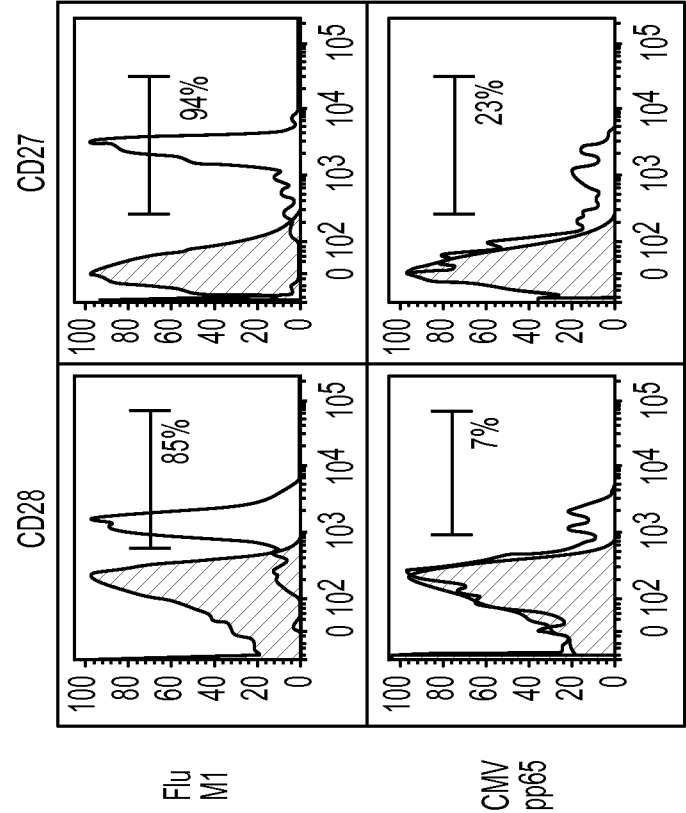


FIG. 9A

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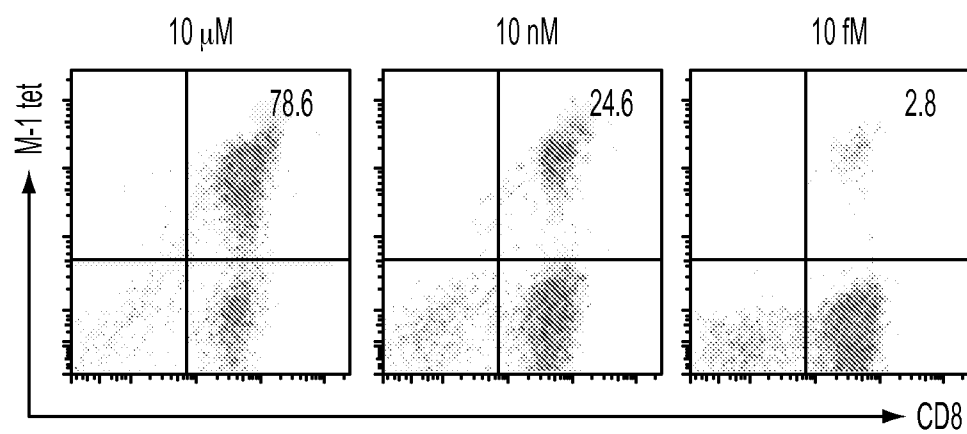


FIG. 10A

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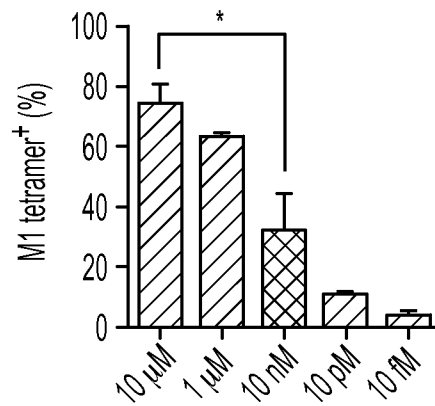


FIG. 10B

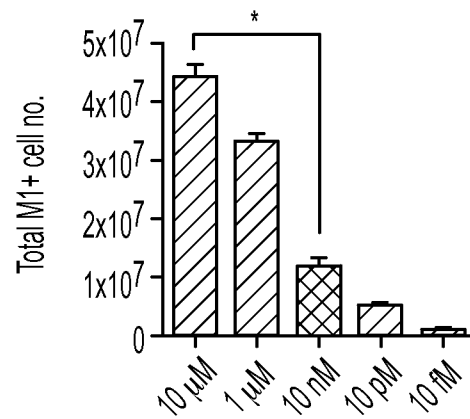


FIG. 10C

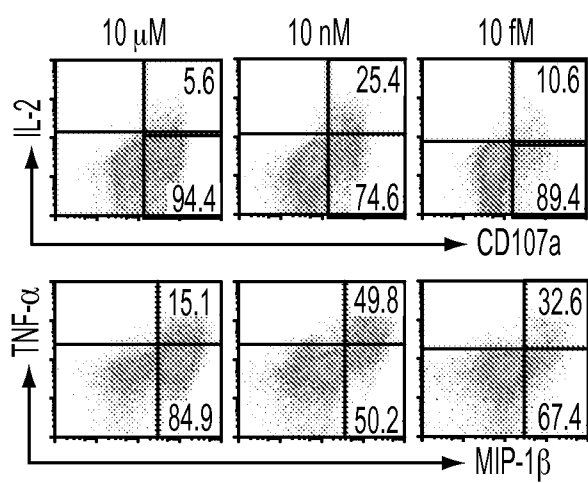


FIG. 10D

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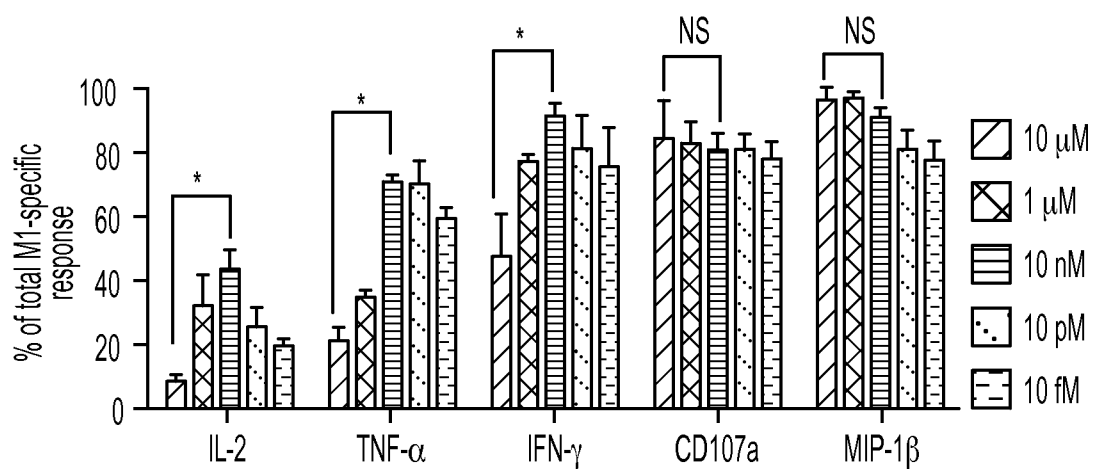


FIG. 10E

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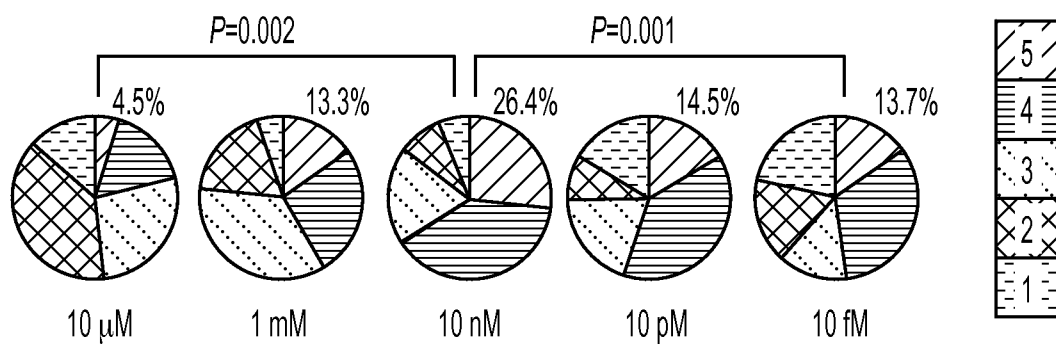


FIG. 10F

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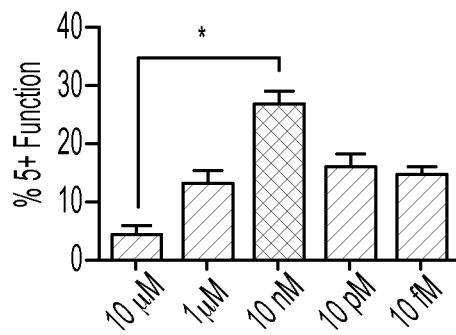


FIG. 10G

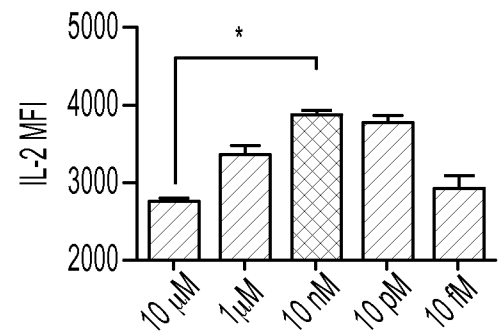


FIG. 10H

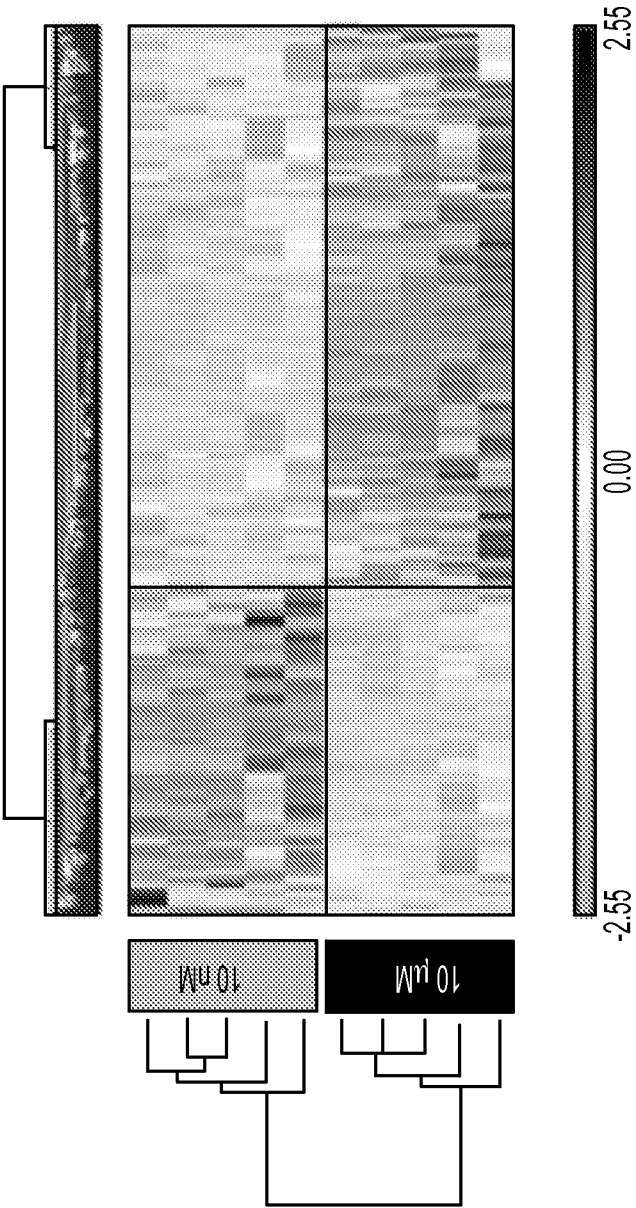


FIG. 11A

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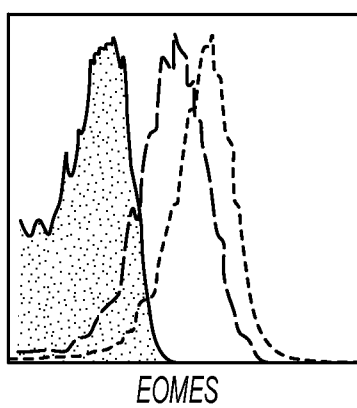


FIG. 11B

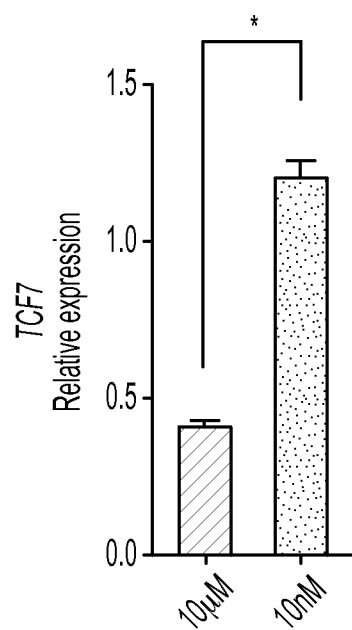


FIG. 11C

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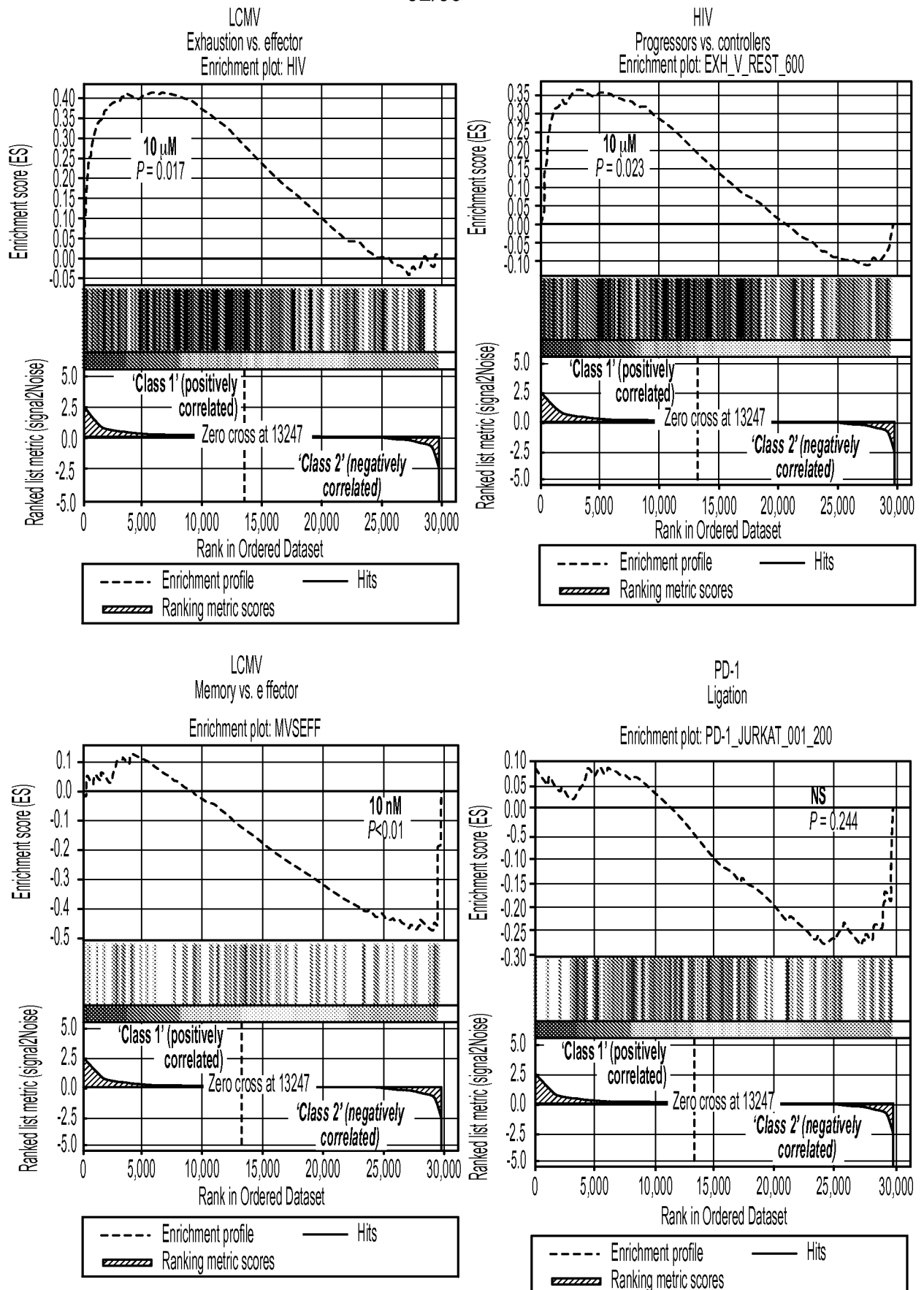


FIG. 11D

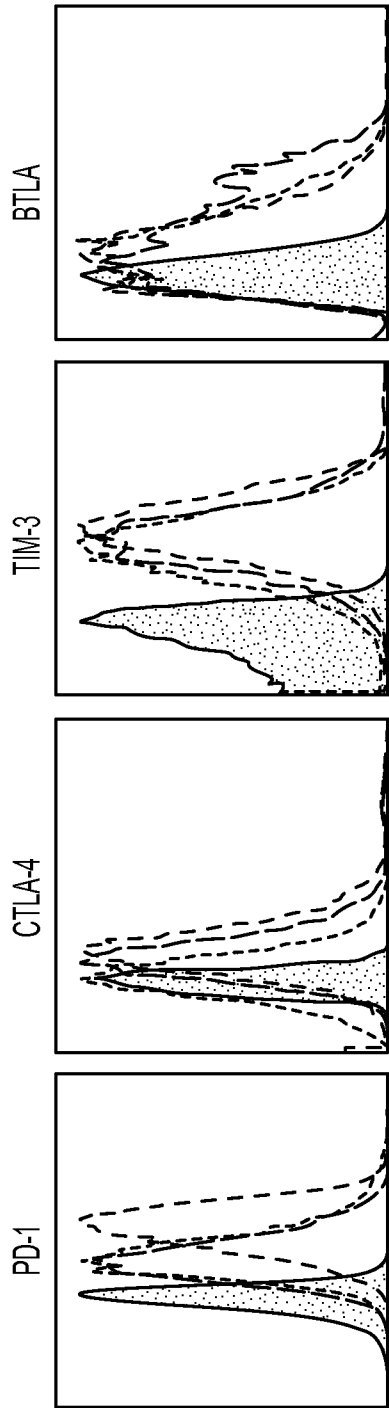


FIG. 12A

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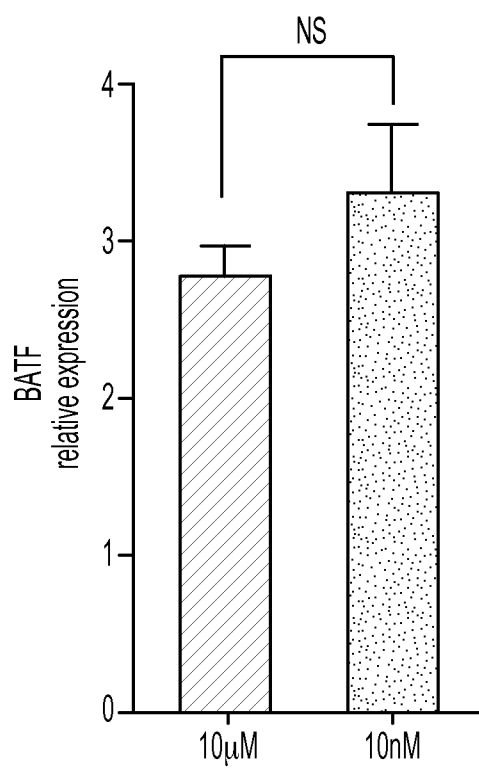


FIG. 12B

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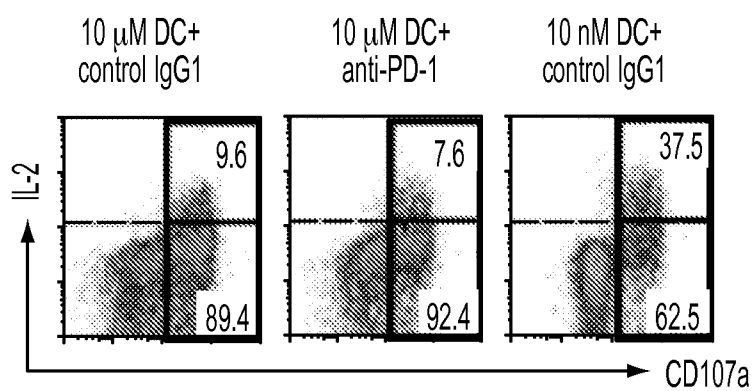


FIG. 12C

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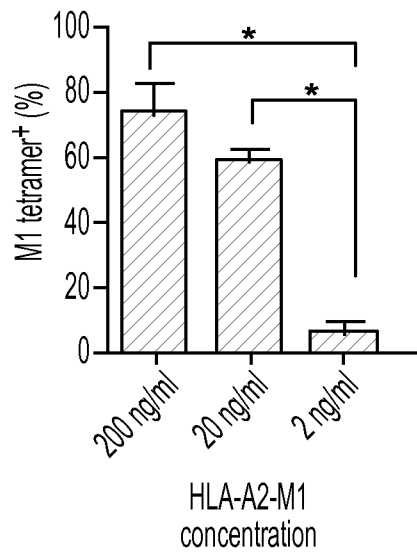


FIG. 12D

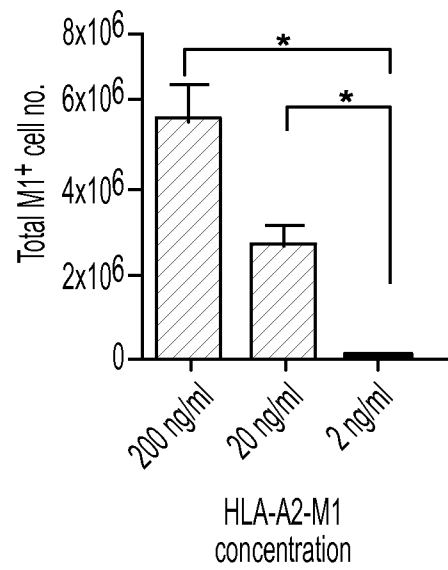


FIG. 12E

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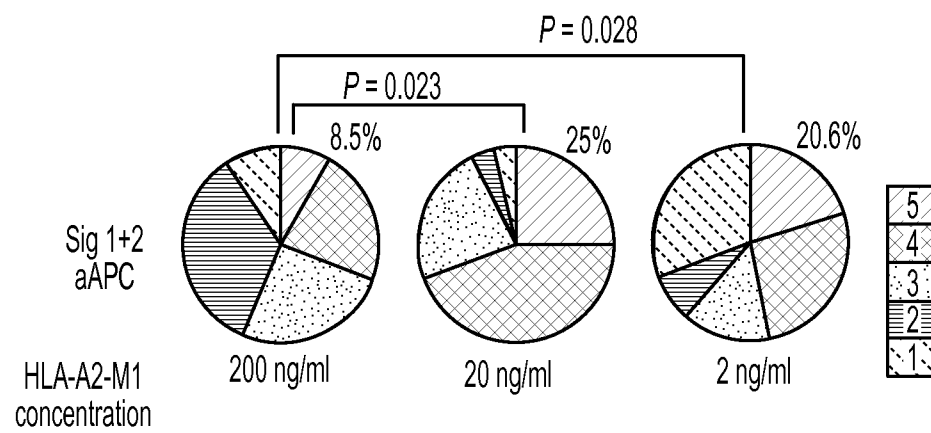


FIG. 12F

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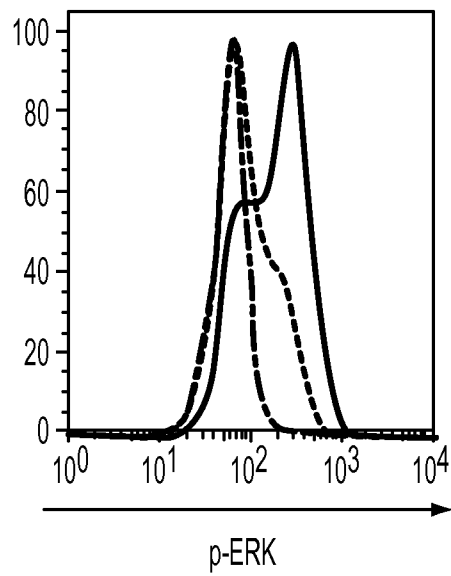


FIG. 13A

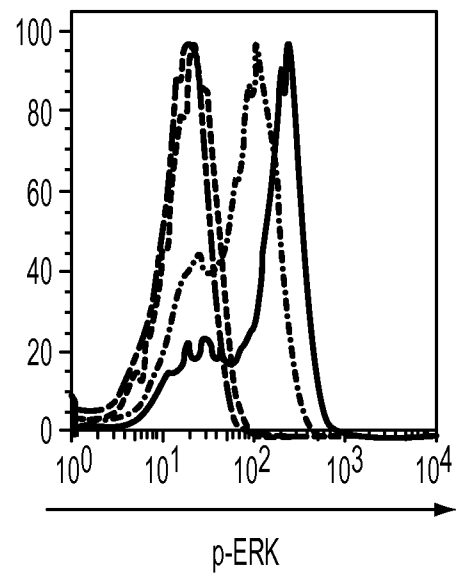


FIG. 13B

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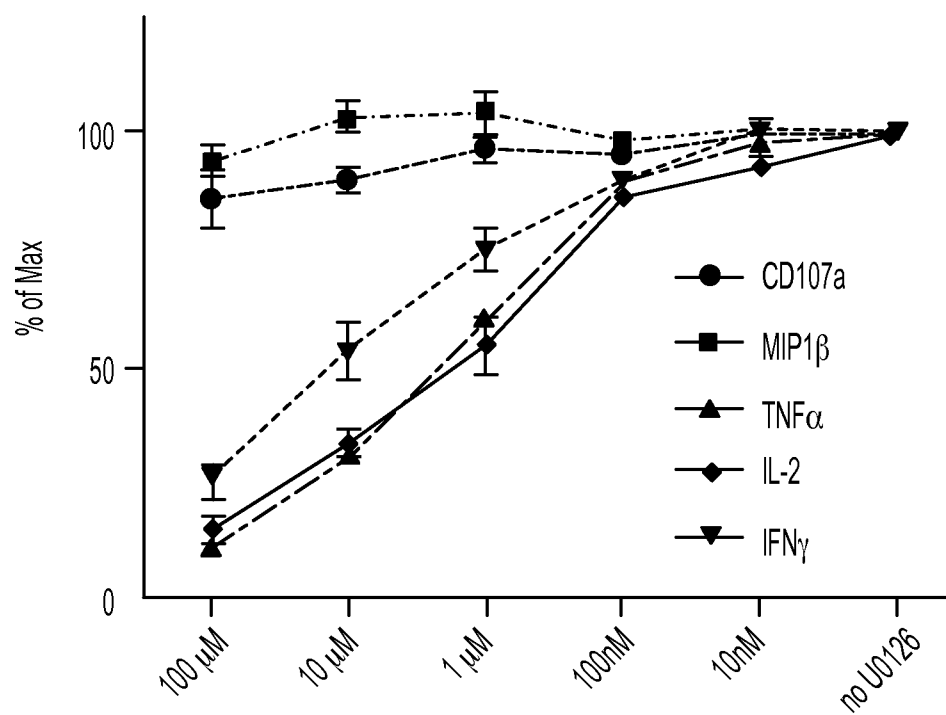


FIG. 13C

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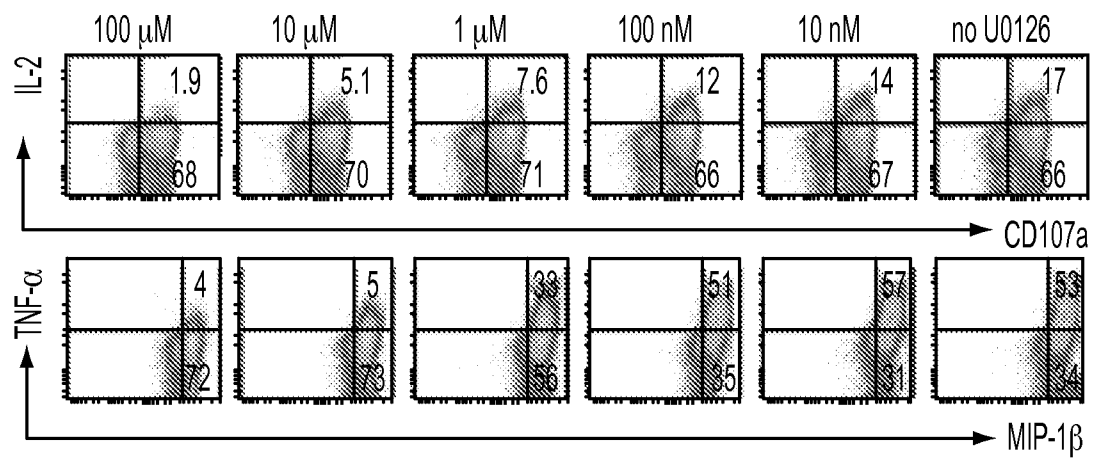


FIG. 13D

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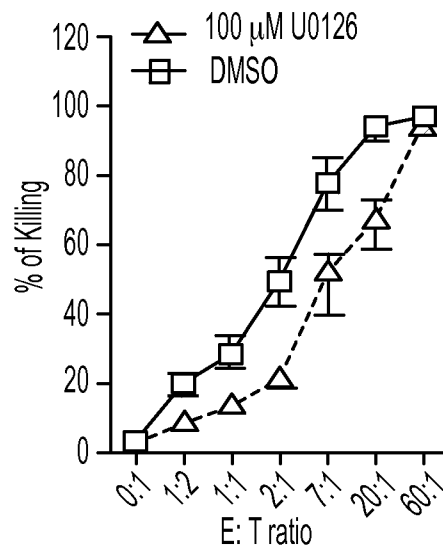


FIG. 13E

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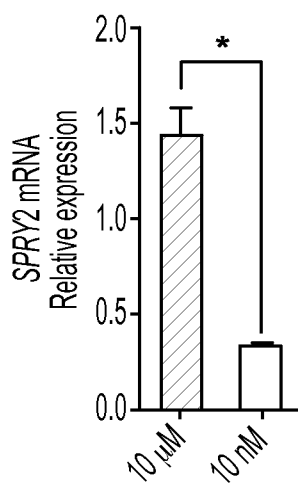


FIG. 14A

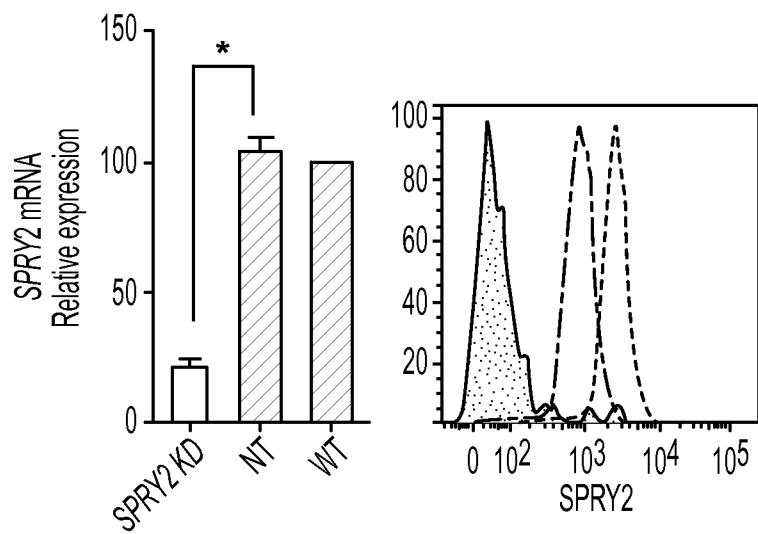


FIG. 14B

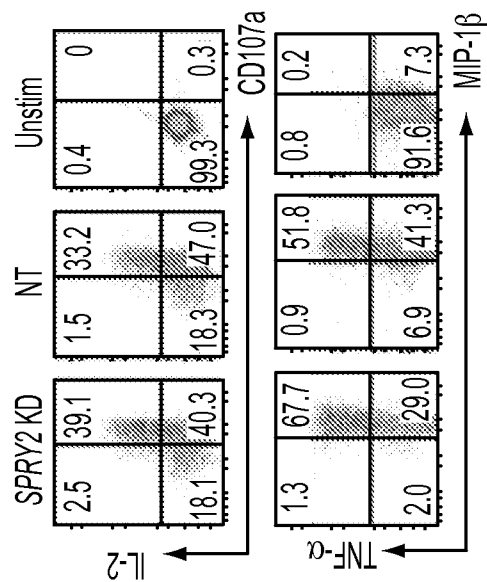


FIG. 14C

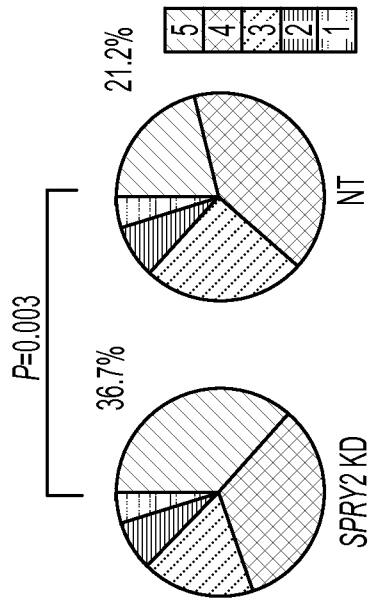


FIG. 14D

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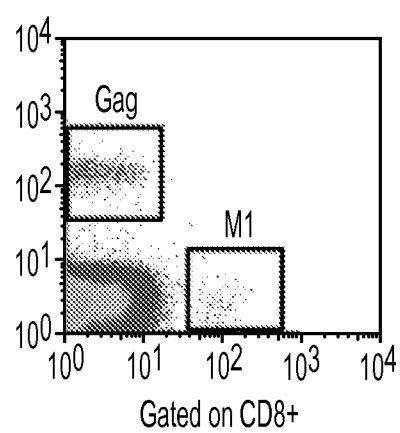


FIG. 15A

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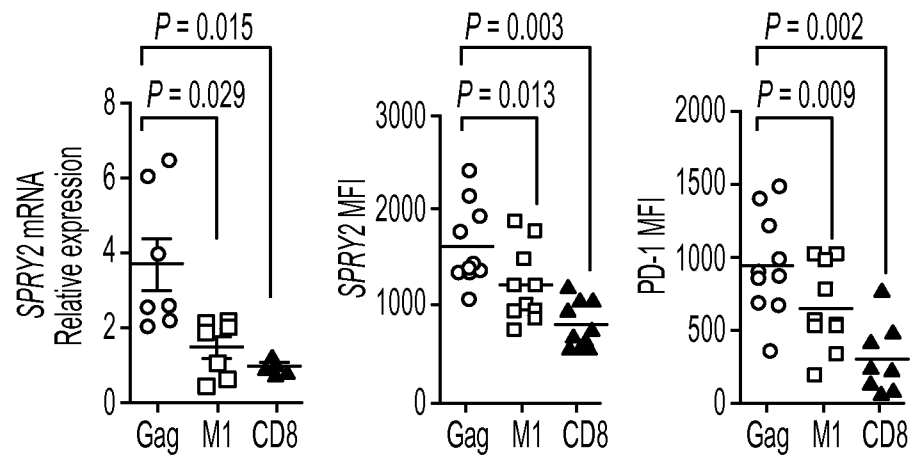


FIG. 15B

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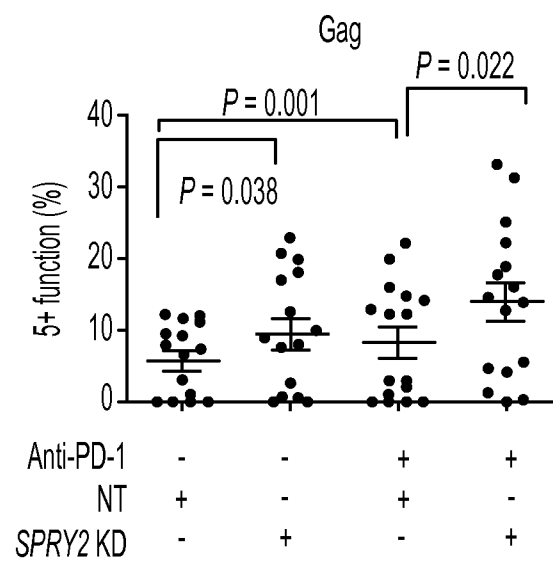


FIG. 15C

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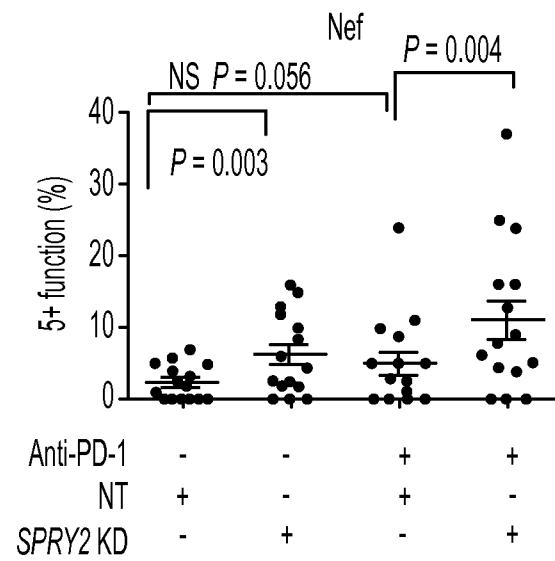


FIG. 15D

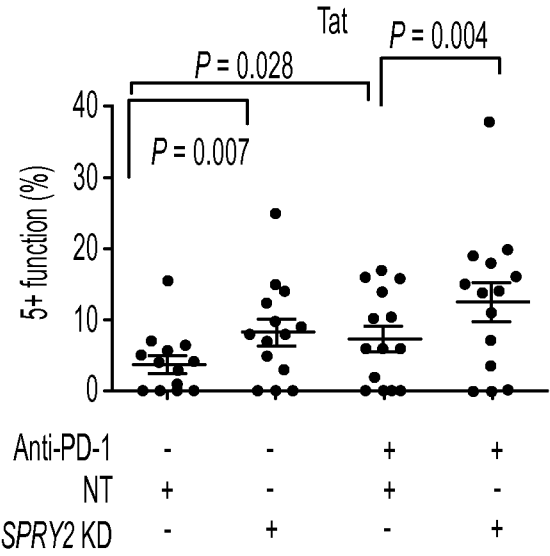


FIG. 15E

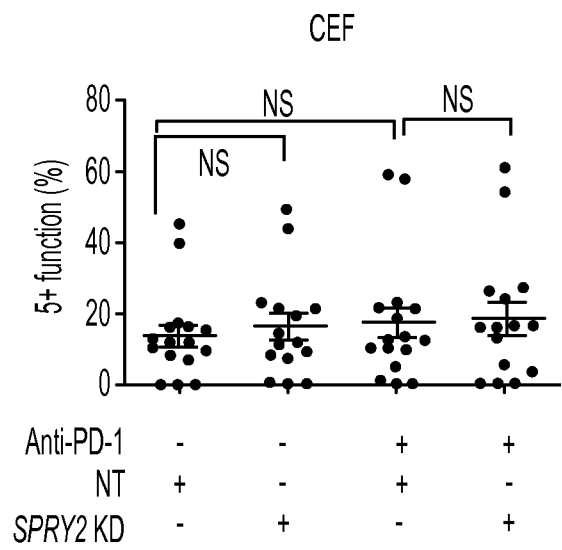


FIG. 15F

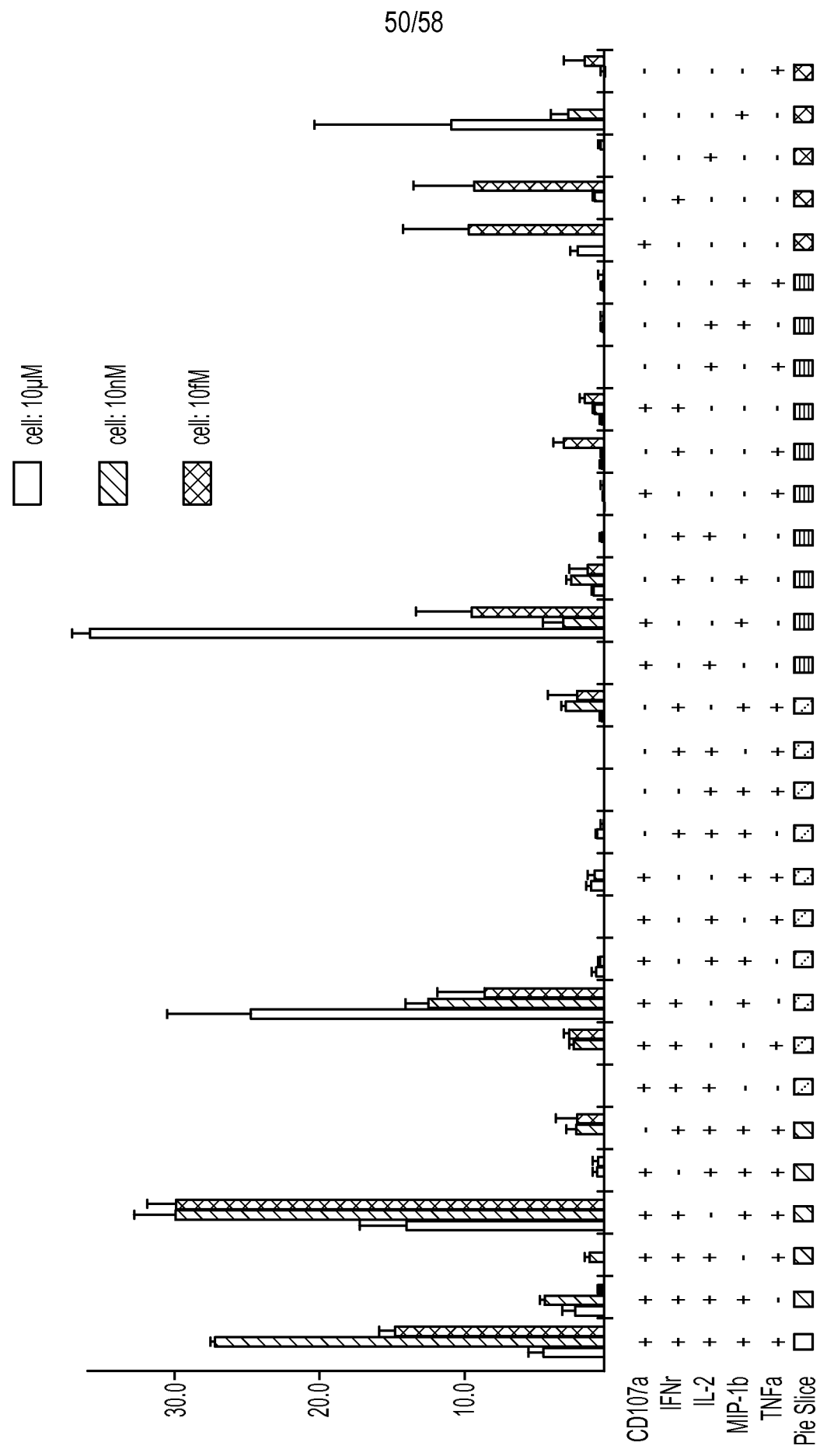


FIG. 16

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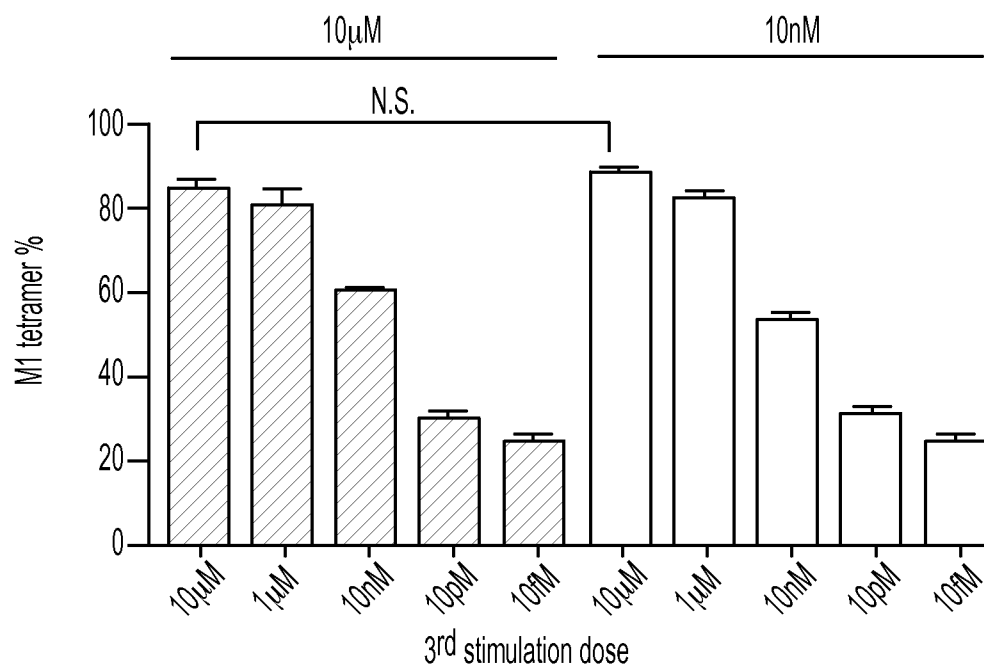


FIG. 17A

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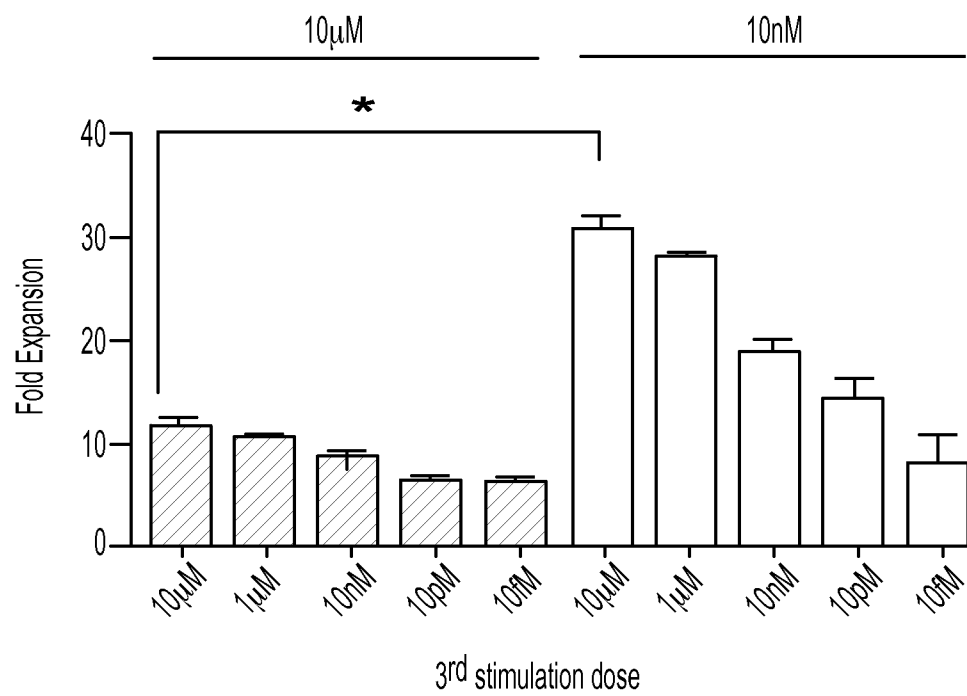


FIG. 17B

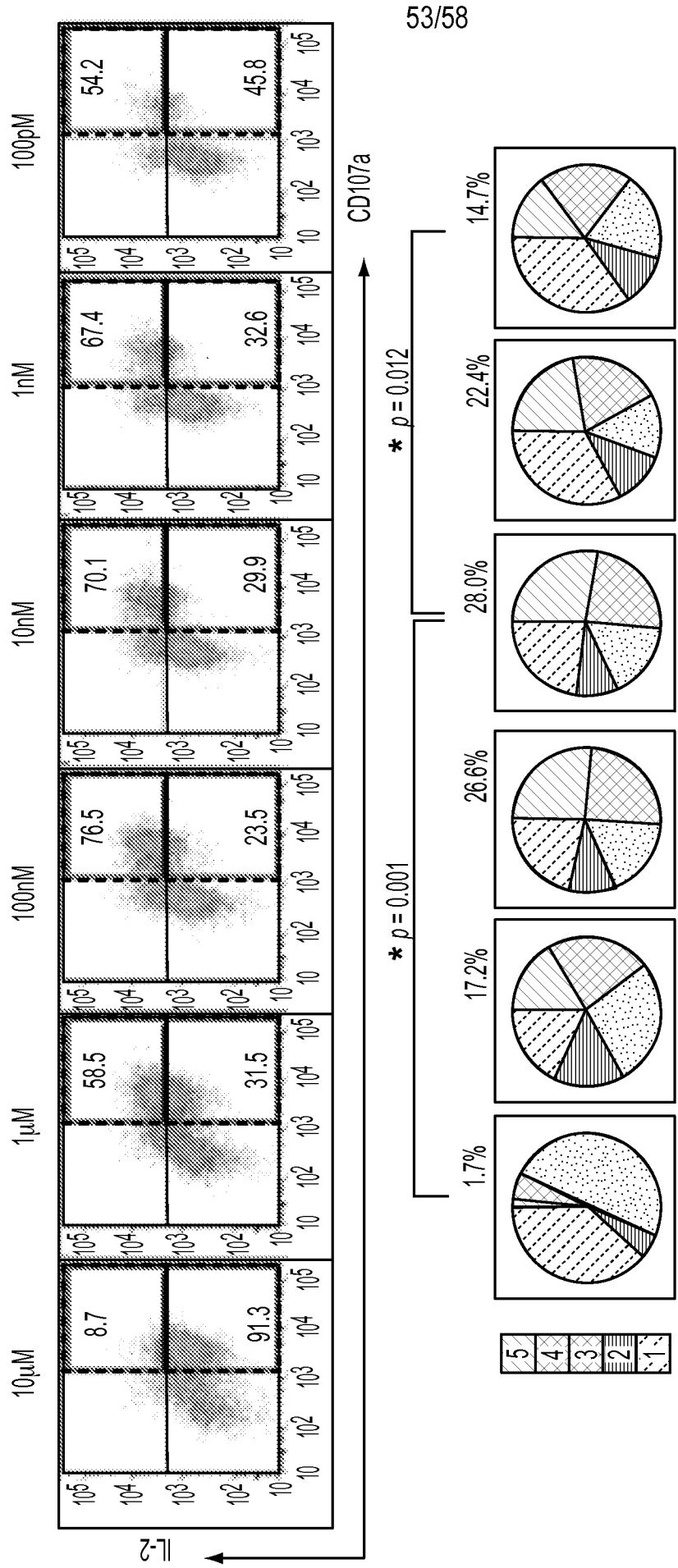


FIG. 18

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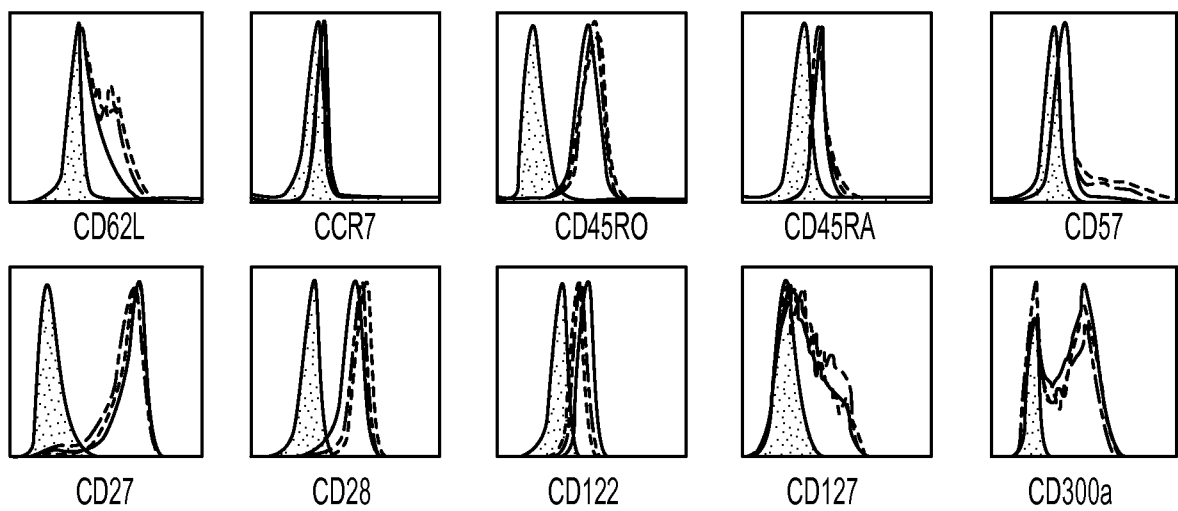


FIG. 19A

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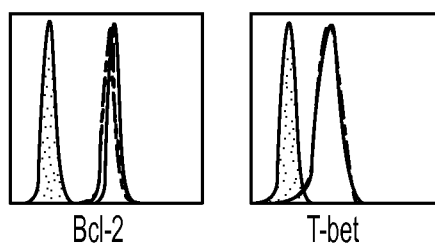


FIG. 19B

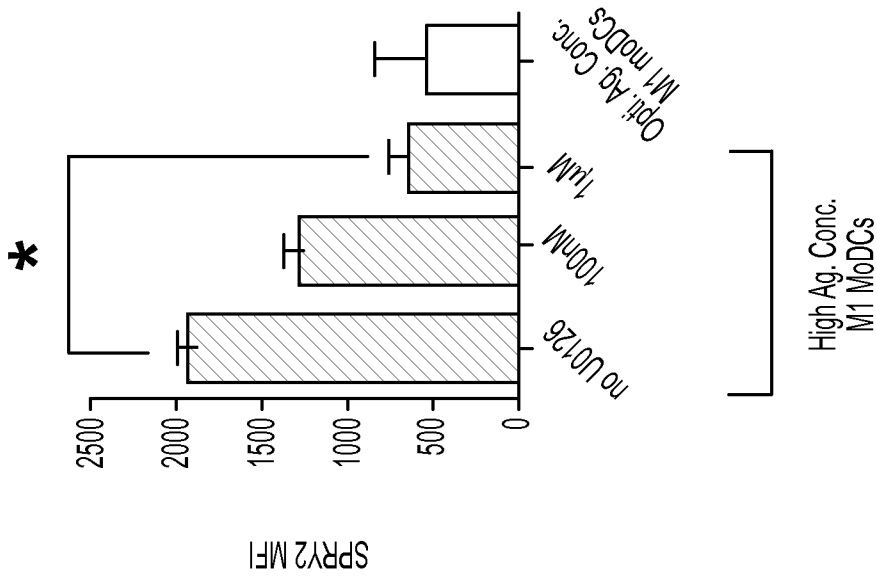


FIG. 20B

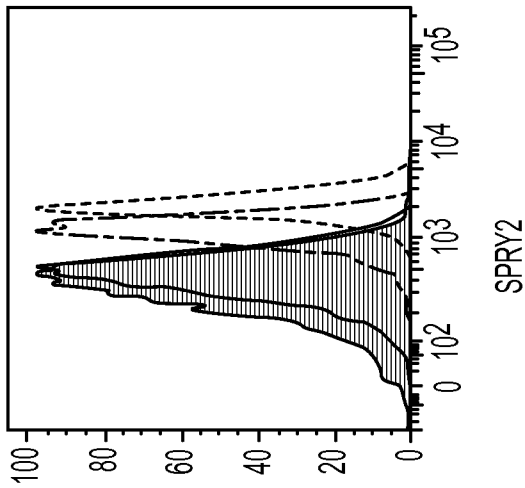


FIG. 20A

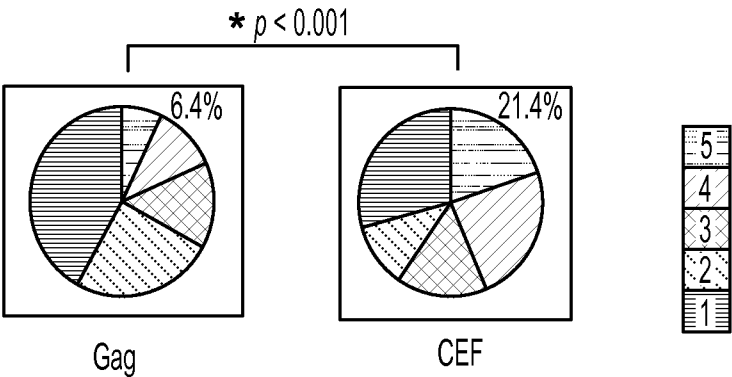


FIG. 21A

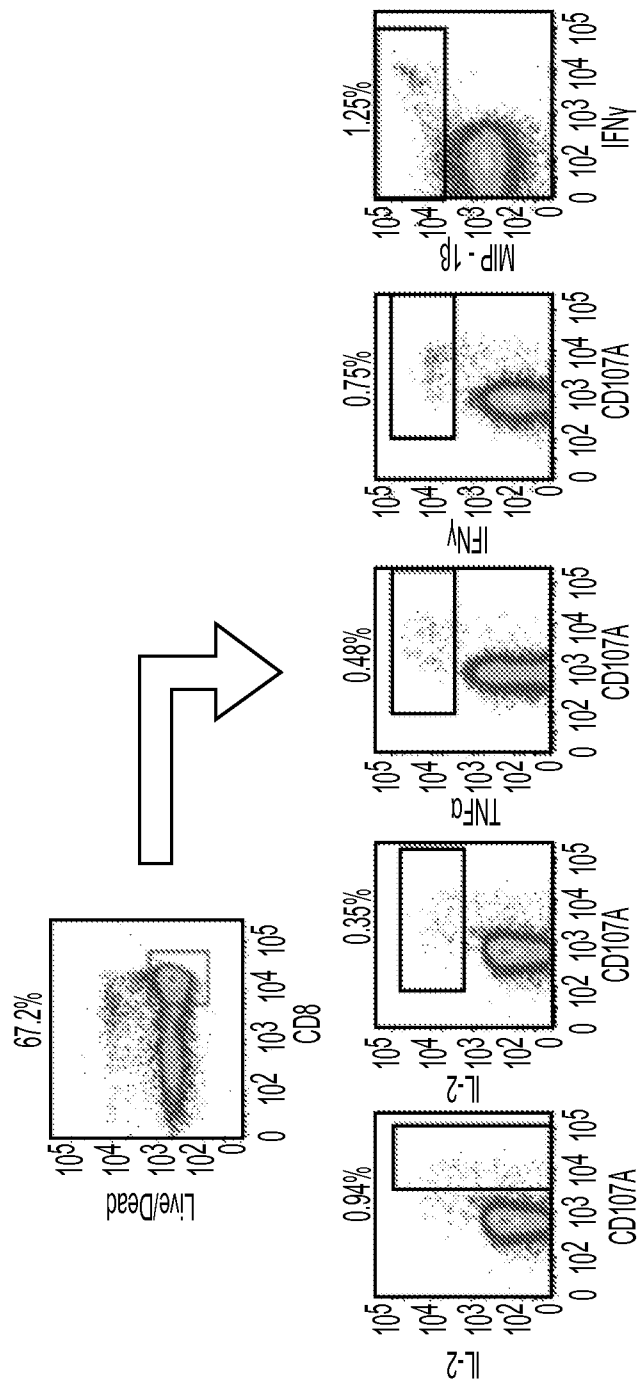


FIG. 21B

A. CLASSIFICATION OF SUBJECT MATTER**C12N 15/113(2010.01)i, C12N 5/0783(2010.01)i, A61K 31/7088(2006.01)i, A61K 38/00(2006.01)i, A61P 35/00(2006.01)i**

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)

C12N 15/113; A61K 31/35; A61K 31/7048; A61K 38/55; A61K 48/00; A01N 43/16; A61K 31/663; C12N 5/0783; A61K 31/7088; A61K 38/00; A61P 35/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean utility models and applications for utility models

Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKOMPASS(KIPO internal) & keywords: T cell, polyfunctionality, SPRY2, Wnt/ β -catenin, chronic infection, cancer**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2007-0066522 A1 (MCCORMICK, J. J. et al.) 22 March 2007	9
A	See abstract; claims 1-27.	1-4, 7-8, 10
X	WO 2010-075286 A1 (UNIVERSITY OF WASHINGTON et al.) 1 July 2010	10
A	See abstract; tables 1-2; claims 1-8.	1-4, 7-9
X	US 2011-0251144 A1 (MOON, R. T. et al.) 13 October 2011	10
A	See abstract; tables 1-3; claims 1-19.	1-4, 7-9
A	SCHENKEL, J. M. et al., `Beta-catenin signaling mediates CD4 expression on mature CD8+ T cells`, The Journal of Immunology, 14 July 2010, Vol. 185, No. 4, pp. 2013-2019.	1-4, 7-10
	See abstract; figures 1-2.	
PX	CHIU, Y. L. et al., `Sprouty-2 regulates HIV-specific T cell polyfunctionality`, The Journal of Clinical Investigation, 2 January 2014, Vol. 124, No. 1, pp. 198-208.	1-2, 7, 9
	See abstract; pages 198-199; figures 1-2.	

☐ 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

26 February 2015 (26.02.2015)

Date of mailing of the international search report

27 February 2015 (27.02.2015)

Name and mailing address of the ISA/KR

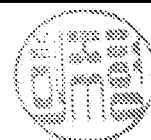
International Application Division
Korean Intellectual Property Office
189 Cheongsu-ro, Seo-gu, Daejeon Metropolitan City, 302-701,
Republic of Korea

Facsimile No. ++82 42 472 3473

Authorized officer

HEO, Joo Hyung

Telephone No. +82-42-481-8150



INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US2014/067674

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2007-0066522 A1	22/03/2007	US 2009-0093432 A1 US 7514549 B2 US 7645871 B2 WO 2006-113579 A2 WO 2006-113579 A3	09/04/2009 07/04/2009 12/01/2010 26/10/2006 12/07/2007
WO 2010-075286 A1	01/07/2010	US 2012-0046242 A1 WO 2010-075286 A8	23/02/2012 04/11/2010
US 2011-0251144 A1	13/10/2011	US 2014-0080775 A1 WO 2010-033581 A1	20/03/2014 25/03/2010

INTERNATIONAL SEARCH REPORTInternational application No.
PCT/US2014/067674**Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 5-6
because they relate to subject matter not required to be searched by this Authority, namely:
Claims 5-6 pertain to methods for treatment of the human body by therapy, and thus relate to a subject matter which this International Searching Authority is not required, under PCT Article 17(2)(a)(i) and PCT Rule 39.1(iv), to search. .
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Group 1, claims 1-2, 7 and 9, directed to SPRY2.

Group 2, claims 3-4, 8 and 10, directed to Wnt/ β -catenin signaling pathway.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.