Title

Enhancing antigen cross-presentation in human monocyte-derived dendritic cells by recruiting the intracellular Fc receptor, TRIM21[1]

Running Title

Using TRIM21 to enhance dendritic cell cross-presentation

Authors

Patricia ML Ng*, Nivashini Kaliaperumal†, Chia Yin Lee*, Wen Jie Chin*, Hwee Ching Tan*, Veonice B. Au†, Angeline X-H Goh*, Qiao Wen Tan*,‡, Darren SG Yeo*,‡, John E Connolly†, Cheng-I Wang*

Affiliation

*Singapore Immunology Network, A*STAR, S138648, Singapore

†Institute of Molecular and Cell Biology, A*STAR, S138673, Singapore

‡School of Life Sciences and Chemical Technology, Ngee Ann Polytechnic, S599489, Singapore.

Corresponding Authors

Cheng-I Wang,

phone number: +65 64070083, e-mail : Wang_ChengI@immunol.a-star.edu.sg

John E Connolly,

phone number: +65 6586 9652, e-mail : jeconnolly@imcb.a-star.edu.sg

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Abstract

Sub-optimal immune responses to pathogens contribute to chronic infections. One way to improve immune responses is to boost antigen presentation. Here, we investigate the potential of the Tripartite Motif-containing 21 (TRIM21) pathway. TRIM21 is a ubiquitously-expressed cytosolic protein that recognizes the Fc region of antibodies. When antibodies that are bound to pathogens enter the cell as immune complexes, binding of TRIM21 to Fc initiates downstream inflammatory signaling and targets the immune complexes for proteasomal degradation. In antigen-presenting cells, peptides generated by proteasomes are loaded onto MHCI molecules to stimulate CD8 T cell responses which are crucial for effective immunity to pathogens. We hypothesized that increasing the affinity between immune complexes and TRIM21 might markedly improve CD8 T cell responses to antigens processed by the TRIM21 pathway. Using phage display technology, we engineered the human IgG1 Fc to increase its affinity for TRIM21 by 100-fold. Adenovirus immune complexes with the engineered-Fc induced greater maturation of human dendritic cell (DC) than immune complexes with unmodified Fc, and stimulated increased antigen-specific CD8 T cell proliferation and interferon-γ release in co-cultures of DC-PBMC (peripheral mononuclear blood cells). Thus by increasing the affinity between Fc and TRIM21, antigens from immune complexes undergo enhanced cross presentation on DC, leading to greater CD8 T cell responses. Given that stimulation of CD8 T cells is both central to defense against intracellular pathogens and an enduring challenge for vaccine design, our study reveals a practical approach to increasing cytotoxic T cell responses to antigens targeted by Fc-modified antibodies.

Introduction

Tripartite motif-containing 21 (TRIM21) is a highly-conserved, ubiquitously-expressed cytosolic protein. It defends against pathogenic agents that enter the cytosol as immune complexes. When adenoviruses infect a cell as an immune complex and penetrates into the cytosol, TRIM21 binds the Fc region of immune complexes. This leads to TRIM21 ubiquitination, which targets the immune complexes for proteasomal degradation (1). TRIM21-mediated pathogen degradation in non-immune cells has been shown for adenoviruses (1), human rhinovirus (2), picornaviruses (3), *Salmonella* (4), and the Alzheimer's Disease pathogenic protein, Tau (5), and is termed "antibody-dependent intracellular neutralization" (ADIN) (6). During degradation by the proteasome, TRIM21 releases free ubiquitin chains that initiate pro-inflammatory signaling through NFκB, AP-1 and IRF signaling pathways (7, 8). Hence TRIM21 sets the cell into an anti-pathogen state by mediating pathogen degradation and triggering pro-inflammatory pathways.

Alongside ADIN, the generation of pathogen-derived peptides during proteasomal degradation could have important immune consequences, especially in dendritic cells (DC). Mammalian cells routinely present proteasome-generated peptides on major histocompatibility complex I (MHCI) for screening by CD8 T cells; when these T cells recognize the peptides, they kill the infected cell and thereby control infections. For this to occur, CD8 T cells must first be stimulated by recognition of their cognate peptide presented by MHCI on an activated DC (9). This process is called cross-presentation. Induction of effective immunity following vaccination relies on efficient cross-presentation of the exogenous vaccine antigen by DC to stimulate optimal CD8 T cell responses. However, vaccines that activate an effective CD8 T cell response remain challenging to design.

Given that TRIM21 mRNA levels rise during the differentiation of monocytes to DC (10); that TRIM21 mediates degradation of immune complexes (1) in non-immune cells; and that TRIM21 regulates the inflammatory pathways (7, 11), we asked whether TRIM21 could be exploited to enhance DC cross-presentation of pathogenic antigens to CD8 T cells.

In HEK293T cells, NF-κB signaling is drastically reduced when the affinity of Fc for TRIM21 is reduced (12). Conceivably, the affinity between the Fc region of antibodies and TRIM21 dictates the duration that the ubiquitin ligase in the RING domain of TRIM21 remains active. A higher affinity allows a longer duration for the formation and extension of K63-linked ubiquitin chains on TRIM21 itself. These ubiquitin chains, when released from TRIM21, would activate protein kinases such as TAK1(13), which in turn triggers the signaling of

downstream pro-inflammatory pathway. Based on this, we hypothesize that increasing the affinity of Fc for TRIM21 will increase the stimulation of pathways leading to DC maturation. Here, we engineered the Fc region of antibodies to increase its affinity for TRIM21 by 100-fold. This led to immune complexes that promoted DC maturation more strongly. These DC in turn, more strongly stimulated antigen-specific CD8 T cell proliferation and cytokine production. The wider application of this technique holds great promise for vaccine development.

Materials and Methods

PBMCs

PBMCs were isolated from apheresis blood of healthy donors using Ficoll-Paque Premium (GE Healthcare). Use of apheresis blood for this study was approved by the National University of Singapore Institutional Review Board.

MoDCs

PBMCs were used for the isolation of monocytes using CD14 Microbeads (Miltenyi). Monocytes were differentiated into moDC using 100ng/ml of GM-CSF (premium grade, Miltenyi) and 100ng/ml of IL-4 (premium grade, Miltenyi) in RPMI-1640 with 25mM HEPES and L-Glutamine (Hyclone) that is further supplemented with 10% FBS (South American origin, Gibco) and antibiotics comprising 100U/ml Penicillin and 100ug/ml Streptomycin (Gibco). On the fourth day of the culture, a third of the medium is replaced with fresh medium with the same supplements. After 7 days, moDC (floating cells) from the cell culture were harvested for experiments.

Cell lines

HEK293T cells and HeLa cells were maintained in 1g/L glucose DMEM (Hyclone) that is supplemented with 10% FBS (South American origin, Gibco) and antibiotics (100U/ml Penicillin, 100ug/ml Streptomycin, Gibco).

Viruses

Purified E1- and E3- deleted replication-deficient adenovirus type 5 with the eGFP reporter gene (VQAdCMV eGFP, ViraQuest) was used for the ADIN assays. The same strain of adenovirus with no eGFP gene (VQAd EMPTY, ViraQuest) was used for all other assays. To titer the virus, HEK293T cells were seeded onto 0.01% poly-L-lysine (Sigma) coated 24-well plates at a cell density of 2.5×10^5 cells/ml. Once the cells had adhered, 10-fold serial dilutions of viruses were added to the cell culture and incubated for 2 days. Viral titer was calculated from the number of infected cells as detected by the Adeno-X Rapid Titer Kit (Clontech).

Human TRIM21 (PRYSPRY domain) protein.

Human recombinant TRIM21 PRYSPRY domain (amino acid position 287-475) was cloned into the 3'-end of a His-tag in the pQE-2 bacteria expression vector and expressed in *E. coli* BL21 (DE3). A 20 ml overnight culture was inoculated into 380 ml fresh medium and grown at 37°C until OD_{600} reaches 1.0. Protein expression is induced with 1 mM IPTG for 4.5 hours at room temperature. The expressed protein was then purified from cell lysates using

Ni-NTA resin (Qiagen) followed by gel filtration in PBS in a Superdex 75 chromatography column (GE Healthcare).

Antibodies

The variable domains of the chimeric anti-adenovirus antibody were cloned from hybridoma 9C12 (TC31-9C12.C9) obtained from the Developmental Studies Hybridoma bank (NICHD/ University of Iowa). The variable domains of the human anti-DEC205 antibody were synthesized by GenScript Biotech based on the published sequence of clone3G9-2D2 (US patent 8,236,318 B2). The variable domains were cloned into the N-terminal of the constant domains of the human IgG1 in the pTT5 vector. For the Fc-modified antibodies, the 5 amino acid modifications in the constant regions are modified by using the Quikchange Lightning Multi Site-Directed Mutagenesis Kit (Agilent Technologies). Antibodies were expressed in HEK293-6E in our lab or in CHO cells by WuXi AppTec, and purified from their supernatants using Protein G beads. The purified antibodies were buffer-exchanged into 20 mM His, 150 mM NaCl, pH 6.0, then filtered and assayed for endotoxin content using the Limulus Amoebocyte Lysate–QCL1000 (Lonza). Only antibody preparations with an endotoxin content of <1EU/μg were used for cell-based assays.

Peptides

Peptides were synthesized by GenScript Biotech according to the following sequences: TYFSLNNKF (HLA-A*2401-Ad5 epitope), YVLFEVFDVV (HLA-A*0201-Ad5 epitope), LAVFEDYVAF (scrambled peptide) and SLYNTVATL (HLA-A2-HIV epitope).

Phage display library construction

The PN04-44AD phagemid has the human IgG1 Fc gene encoding amino acid positions 239 to 447 (EU numbering) fused to an amber stop (TAG) followed by a truncated gIII. PN04-44AD was used as the template for making the phage display library using a Kunkel reaction-based site-directed mutagenesis strategy that was previously described (14): briefly, a single-stranded template was used for annealing of oligonucleotides that carry the library sequences; double-stranded DNA was then produced and electroporated into *E. coli* TG-1 cells that were pre-infected with helper phage. The electroporated TG-1 cells were enumerated by plating dilutions of an aliquot onto 2YT plates with ampicillin and kanamycin. The remaining electroporated TG-1 were cultured at 37°C, overnight, in 2YT medium with ampicillin and kanamycin for the production of bacteriophages. The next day, the bacteriophage were harvested from the supernatant of the culture and stored in PBS with 20% glycerol until use.

Biopanning of the phage display library

Recombinant human TRIM21 PRYSPRY domain was biotinylated and captured onto DynaBeads M-280 Streptavidin (Invitrogen) for use as bait for biopanning. In the first round of biopanning, 2 x 10^{12} bacteriophage were incubated with streptavidin beads that had been pre-incubated with 5 nM of bait protein. The beads were then washed five times with casein, incubated for 30 mins with 1 μM of soluble Fc and eluted using trypsin. In the second round of biopanning, the eluted bacteriophage were amplified and incubated first with empty streptavidin beads, and then with streptavidin beads that had been pre-incubated with 0.5 nM of bait protein. The beads were washed ten times with casein and incubated with 1 μM soluble Fc for two rounds of 30 mins before elution using trypsin. In the third round of biopanning, the eluted phage were amplified, incubated with empty streptavidin beads, and then with streptavidin beads that had been pre-incubated with 0.05 nM of bait protein. After incubation, the beads were washed ten times, then incubated for five hours with 1 μM of soluble Fc before elution using trypsin. Fifty clones from each round of round two and three were sequenced.

Surface Plasmon Resonance (SPR) analysis

The affinity constants were measured using ProteON XPR36 (Bio-Rad). Antibodies were immobilized onto a GLC sensor chip via amine coupling, and PBS with 0.05% Tween-20 was used as the running buffer. Curves were fitted with the ProteON Manager software using five concentrations of TRIM21 and based on a Langmuir 1:1 model.

Antibody-dependent intracellular neutralization (ADIN) assays

For ADIN assays, 1.25 x 10^5 HeLa cells were seeded into 24-well plates and 1 x 10^5 moDC were seeded into 96-well plates for infection with immune complexes, virus alone, antibodies alone or medium alone for 48 h. As HeLa cells are highly permissive to adenovirus infection (15), an MOI of 1.2 was used, while for moDC an MOI of 400 was used. Infected cells were detected by expression of eGFP using flow cytometry. For HeLa cells, immune complexes were pre-formed by incubation of virus with antibodies at concentrations ranging from 0.3 pM to 35 nM for 1 h at room temperature prior to use. Thereafter, the incubation mixture is diluted by 55-fold into the HeLa cell culture to give a final concentration ranging from 0.006 pM to 627 pM. For moDC, immune complexes were pre-formed by incubation of virus with antibodies at concentrations ranging from 0.17 nM to 690 nM for 1 h at room temperature prior to use. Thereafter, the incubation mixture is diluted by 12.5-fold into the moDC culture to give a final concentration ranging from 0.01nM to 55 nM.

MoDC maturation assay

1x10^5 moDC were incubated in 200 µl of medium in 96-well plates with either PBS, 4x10^7 Ad5 virus, 1.6 µg antibodies or immune complexes made from 4x10^7 Ad5 virus and 1.6 µg antibodies that were pre-incubated for 1 h at room temperature. All treatments were administered to the cell culture in a volume of 8 µl. For a positive control, 1 µg/ml LPS was used. The cells were analyzed by FACS after 24 h.

MoDC: autologous $CD14^-$ PBMC co-culture assay

MoDC were prepared and treated in the same way as in the moDC maturation for 4 h. After 4 h, the cells were co-cultured in fresh medium with 5x10^5 autologous $CD14^-$ PBMCs that had been labeled with 10µM CFSE using the Vybrant™ CFDA SE Cell Tracer kit (Life technologies): briefly, 100 million cells were labeled in 1 ml of 10µM CFSE in FBS-supplemented medium for 5 mins at room temperature. Excess dye was then removed by centrifugation at 10,000 x g for 1 min and the cells were washed three times with fresh medium. The co-culture was maintained for up to 13 days with replacement of 1/3 old medium with fresh medium on days 4 and 7. For positive controls, either 1 µg/ml LPS was added to the moDC, as above, or 2 µl T Cell TransACT™ (Miltenyi).

Peptide re-stimulation assays

To prepare moDC for pulsing of peptides, autologous moDC were generated using the same method as above from frozen monocyte stock. After 6 days, moDC were pulsed for a day with either 10 µg/ml of peptides sterile-filtered, or 200 µg Peptide libraries (Miltenyi, PepTivator AdV5 Hexon or PepTivator NY-ESO-1) in medium containing 100 ng/ml GM-CSF (Miltenyi), 100 ng/ml IL-4 (Miltenyi) and 50 ng/ml TNF-α (Miltenyi). Thereafter, 1 x 10^5 of the peptide-pulsed moDCs were used to re-stimulate an autologous 11 day old moDC: $CD14^-$ PBMC co-culture at a ratio of 1:5 peptide-pulsed moDC is to 11 day old co-culture. For donor LCY02, cells were re-stimulated for 16 hours and the supernatant of the re-stimulated cells was harvested for ELISA. The cells were then treated with fresh medium containing 1 µg/ml Brefeldin A. After 5 h, cells were harvested and labeled for flow cytometry analysis. For donor LCY10, cells were re-stimulated for 16 hours in the presence of 1.5 µg/ml Brefeldin A and then harvested for flow cytometry. For the 11 day old moDC: $CD14^-$ PBMC co-cultures, replacement of 1/3 old medium with fresh medium was done at days 4 and 7 for LCY02; and for LCY10, feeding was done on day 4 followed by dilution of the co-culture into an equal volume of fresh medium containing 10ng/mL IL-7 and IL-15 on days 7 and day 10.

Flow cytometry

In all assays, LIVE/DEAD™ Fixable Aqua Dead Cell Stain Kit (Invitrogen) was used to exclude dead cells. In the moDC maturation assay, cells were incubated with anti-FcR-blocking antibody (eBioscience, San Diego, CA, USA) and then labelled using combinations of the following antibodies: Pacific-Blue-anti-CD14 (M5E2), APC-anti-CD11c (S-HCL-3), Alexa Fluor700-anti-CD80 (L307.4), FITC-anti-CD83 (HB15e), PE-Cy7-anti-CD86 (FUN-1), APC-Cy7-anti-HLA-DR (L243), BV650-anti-CCR7 (G043H7) and PE-anti-CD206 (19.2). In the co-culture assay, cells were incubated with human FcR blocking reagent (Miltenyi) and then labelled using the following antibodies: Alexa Fluor647-anti-CD3 (SK1), PE-Cy7-anti-CD4 (OKT4), Pacific Blue-anti-CD8 (SK7), and PE-anti-CD56 (AF12-7H3). In the haplotyping of donors, the dyes used were Alexa Fluor 647-anti-HLA-A24 (17A10) and PE-anti-HLA-A2 (BB7.2). For haplotype controls, a HLA-A24+ cell line HT29 and HLA-A2+ cell line MDA-MB-231 were labeled alongside. In the re-stimulation assays, the dyes used were Alexa Fluor647-anti-CD3 (SK7), PE-Cy7-anti-IFN-γ (4S.B3), Pacific Blue-anti-CD8 (SK1), and PE-anti-CD56 (AF12-7H3). After the surface markers are labeled, cells were fixed and permeabilized using BD Cytofix/Cytoperm solution followed by PE-Cy7-anti-IFN-γ. For absolute cell counts 10 µl of CountBright™ Absolute Counting Beads were added to cells. Samples were acquired using BD FACSDiva software on the LSRFortessa cell analyzer and FACSCanto II (BD Biosciences). Data were analyzed using FlowJo software (Tree Star, Ash-land, OR, USA).

Cytokine and chemokine analysis

Supernatants from the moDC maturation assays were analyzed by a multiplex analysis using human cytokine/chemokine bead panel 1 and 2, which measure a total of 64 targets (Milliplex MAP kits, Millipore) on a Flexmap 3D system (Luminex Corp, Texas, USA). Supernatants from the co-culture assays were analyzed by ELISA for the level of IFN-γ using Human IFN-γ ELISA MAX™ Standard (Biolegend).

Statistical analysis

Statistical analysis were done using ordinary ANOVA in the GraphPad Prism 7.01 software to compare the results from treatment using Fc-modified immune complex with all other treatments. The p-value was corrected for using Dunnett's multiple comparison test. LPS and TransACT were not included in statistical analysis. Where the mean of each treatment is compared to the mean of all other treatments, Tukey's multiple comparison test was used to adjust the p-value.

Results

A modified Fc exhibits increased affinity for TRIM21

To engineer the affinity of Fc for TRIM21, we first identified the amino acids in Fc that are in contact with TRIM21, based on the crystal structure of human immunoglobulin G (IgG) Fc-TRIM21 complex (Fig. 1A) (16). Both the CH2 and CH3 regions of Fc interact with TRIM21. Using a cut-off distance of 5Å, we identified 21 potentially-interacting amino acids on Fc (Supplemental Table 1). The majority of the amino acids interacting with TRIM21 in the CH2 domains were in the α-helix, which could be destabilized if modified; therefore we focused on the amino acids in the CH3 domains, and selected seven whose side groups were nearest and facing towards TRIM21 for modification (Fig. 1B).

We generated a library of 2 billion bacteriophages displaying the human IgG1 Fc with randomly-substituted amino acids in the seven selected positions (Fig. 1B). Recombinant human TRIM21, PRYSPRY domain was used as bait for biopanning. After three sequential rounds of biopanning, we sequenced approximately 50 clones from the output of Round 2 and 3. One sequence was found in ~10% of the clones analysed in Round 2; and in 94% of the clones analysed in Round 3 (Fig. 1C, Clone #1). This variant possessed five amino acid modifications: H433T, N434R, Y436F, S440I and T256P, the last of which was a spurious mutation in the CH2 domain.

To test if the antibodies bearing the modified Fc bound TRIM21 with higher affinity than antibodies without the modifications. Two sets of antibodies were constructed: the first set has variable domains of human antibody, 3G9-2D2 (17) which recognizes human DEC-205, joined to human IgG1 constant regions that have either the modified or unmodified Fc; the second set has variable domains of the mouse antibody, 9C12 (18) which recognizes the Adenovirus Type 5 (Ad5) hexon, also joined to human IgG1 constant regions that have either the modified or unmodified Fc. We termed the first set “human IgG1” and the second set “chimeric IgG1” (Fig. 1D). This will demonstrate if modified Fc could be applied to antibodies with different variable domains and from different species.

Affinity of the two sets of antibodies (Fig. 1D) for TRIM21 was measured using surface plasmon resonance. Modifying the Fc region of human IgG1 increased its affinity for the PRYSPRY domain of TRIM21 by at least 100 fold: the affinity constants (KD) were 0.522 nM for the Fc-modified human IgG1 (Fig. 1E) and 0.425 nM for the Fc-modified chimeric IgG1 (Fig. 1F); while unmodified human (Fig. 1G) and chimeric (Fig. 1H) IgG1 had lower binding affinities (47.3 nM and 125 nM, respectively). Since the crystal structure places T256 out of direct contact with TRIM21, we investigated if the T256P mutation is important for the

317 improved affinity. Converting the proline back to threonine reduced the KD of the Fc-
318 modified chimeric antibody to 23.5 nM (Fig. 1I). The spurious T256P mutation was therefore
319 retained in the Fc-modified antibodies that were used in our subsequent functional studies.
320
321 *Increasing Fc affinity for TRIM21 does not affect ADIN*
322 ADIN was first demonstrated in HeLa cells with adenoviruses (1). The hexon protein of
323 adenoviruses is recognized by the monoclonal antibody 9C12 (18), which we used in the
324 construction of our chimeric antibodies. 9C12 does not block viral entry, but mediates post-
325 entry neutralization, that is dependent on TRIM21(1). Reducing the affinity of antibody for
326 TRIM21 does not affect ADIN of adenovirus in HeLa cells (12). However the effects of
327 increasing the affinity are yet to be tested. We therefore asked if increasing Fc affinity for
328 TRIM21 will affect ADIN in both HeLa cells (non-immune cells) and monocyte-derived DC
329 (immune cells).
330
331 We incubated different concentrations of Fc-modified and unmodified antibodies with
332 replication-defective Ad5 that carries the eGFP (enhanced green fluorescent protein)
333 reporter gene to form immune complexes. These were then added to HeLa cells or
334 monocyte-derived DC (moDC). The frequency of infection was monitored after 48 h via
335 eGFP expression (Fig. 2A). In HeLa cells, antibodies bearing the modified or unmodified Fc
336 regions mediated ADIN of Ad5 infection equally well (Fig. 2B) with an IC50 of 0.9 pM for a
337 viral MOI of 1.2. Similarly in moDC, the IC50 of the unmodified Fc and the modified Fc are
338 very close (Fig. 2C) with an IC50 of 3.0 nM and 4.5 nM respectively for a viral MOI of 400. A
339 higher MOI was used for moDC studies because moDCs were less permissive to infection
340 by adenovirus than HeLa cells. Nonetheless, ADIN mediated by the unmodified Fc and
341 modified Fc remain the same when tested at lower viral MOI (Supplemental Fig. 1). Thus,
342 increasing the affinity of Fc for TRIM21 does not enhance ADIN in both non-immune and
343 immune cells.
344
345 *Increasing Fc affinity for TRIM21 promotes moDC maturation*
346 Unlike ADIN, reducing the affinity of antibody for TRIM21 drastically impaired pro-
347 inflammatory immune signaling in HEK293T cells (12). For moDC, stimulation of T cell
348 requires the simultaneous presentation of cognate antigen, and the provision of activating
349 signals in the form of co-stimulatory molecules on the DC surface and pro-inflammatory
350 cytokines. We therefore asked if increasing Fc affinity for TRIM21 promotes moDC
351 expression of co-stimulatory molecules and pro-inflammatory cytokines.
352

We incubated moDC with an increasing dose of Ad5 and stain for surface markers on moDC which were gated as singlets/live/$CD14^-$, $CD11c^+$ cells using flow cytometry. Results showed no increase in maturation markers (CD80, CD83, CD86, HLA-DR), the migratory marker CCR7, nor a decrease in the endocytic marker (CD206) (Fig. 3A, Supplemental Fig. 2) when moDC were infected by Ad5. This suggest that the viruses are able to suppress moDC maturation as we know that at the highest dose of 400 MOI an average of 69% of the moDC are infected by Ad5 (Fig. 2A, moDC panel). This is in agreement with previous reports that at 50 MOI or higher, E1- and E3- deleted adenoviruses do not upregulate moDC co-stimulatory molecules, and instead suppress responses to LPS or PHA stimulation (19).

Incubating moDC with different dosages of Fc-unmodified Ad5 immune complexes also did not significantly change the surface marker expression (Fig. 3A, Supplemental Fig. 2). At the highest dose of 400 MOI, only moDC treated with Fc-modified immune complexes showed an obvious increase in surface CD80, CD83, CD86 and HLA-DR expression (Fig. 3B). This was dosage-dependent (Fig. 3A, Supplemental Fig. 2) and reproducible in different donors (Fig. 3C)

Increasing Fc affinity for TRIM21 promotes moDC production of Th1-associated chemokines

We next asked if increasing Fc affinity for TRIM21 promotes moDC expression of pro-inflammatory cytokines. Using a multiplex bead-based assay, we measured the secretion of 64 cytokines and chemokines by moDC after 24 h of incubation with Fc-modified or unmodified immune complexes. For each cytokine/chemokine, we calculated the fold change in expression level between treatments with Fc-modified and unmodified immune complexes and correlated these to their dosages (Fig. 4A); this identified nine proteins (CCL-3,-4,-5,-7, CXCL12, TRAIL, IL-6, TNF-α and IFN-γ) with a fold-change to dosage correlation higher than spearman rho 0.3 with $p<0.05$. Comparing the concentrations of these molecules under different moDC treatment conditions using ANOVA revealed that CCL3 (MIP1-α), CCL4 (MIP-1β), CCL5 (RANTES) and TRAIL production was significantly higher following moDC incubation with Fc-modified immune complexes when compared to other treatments (Fig. 4B). Of the remaining five cytokines, IFN-γ, IL-6 and TNF-α also showed a similar trend but the difference at 24 h post-treatment were statistically insignificant.

In vivo, early-maturing DC in peripheral tissues are the main producers of CCL3, CCL4 and CCL5 (20, 21), which attract immature DC and T cells that promote a T helper type 1 (Th1) response (22). Hence, these data suggest that exposure of moDC to Fc-modified immune

complexes induces greater production of Th1-associated pro-inflammatory cytokines in the first 24 h.

Increasing Fc affinity for TRIM21 helps moDC promote CD8 T cell expansion

We next assessed how Fc-modified immune complexes influenced CD8 T cell response in PBMCs. We incubated moDC with either Ad5 alone, or with Fc-modified or unmodified immune complexes, and then co-cultured them with autologous $CD14^-$ PBMCs for 13 days. LPS and TransACT (a CD3/CD28 agonist) were used as positive controls to non-specifically activate DC and T cells respectively.

After 13 days of co-culture, cells were gated by flow cytometry for singlets/live/CD3+CD56- cells to include T cells and exclude NKT cells. In co-cultures where moDC were treated with Fc-modified immune complexes, CD8 T cells were markedly enriched, (Fig. 5A). Furthermore, this enrichment was not due to CD4 T cell death, as the absolute number of CD4 T cells was not lower (Fig. 5B); moreover, the absolute number of CD8 T cells was significantly higher in treatments with the modified Fc immune complexes compared to the other treatments (Fig. 5C).

Analyzing results from 7 different donors, we saw that treatment of moDC with Fc-modified immune complexes significantly increased CD8 T cell counts (Fig. 5E) but not CD4 T cell counts (Fig. 5D) in co-culture experiments when compared to treatment with Fc-unmodified Ad5 immune complexes. Interestingly, there is a heterogeneity in the capacity of cells from different donors to respond to Ad5 and this can be separated into two types: the first type of donors exhibited strongest CD8 proliferation to virus alone (Fig. 5F); while the second type of donors responded poorly to virus alone but showed a marked and significant increase of 5-fold when moDC were treated with the Fc-modified immune complexes (Fig. 5G).

During T cell expansion, IFN-γ is produced. We therefore measure secretion of IFN-γ at 4, 7 and 13 days of co-culture for each donor. As in the CD8 T cell response, treating moDC with Fc-modified immune complexes induced significantly higher IFN-γ production in the moDC: CD14- PBMC co-culture than did either virus alone or Fc-unmodified immune complexes at 4, 7 and 13 days post infection for the Type 2 donors (Fig. 5H).

Combining IFN-γ results from the same 7 donors reveal the same heterogeneity in response for the Type 1 and Type 2 donors (Fig. 5I). Type 1 donors show higher IFN-γ in control treatments and do not increase much more in treatments with Fc-modified immune

complexes. Type 2 donors show much lower IFN-γ level in control treatments and show an increase in treatment with Fc-modified immune complexes.

Thus, by modifying Fc for improved affinity to TRIM21, we generated immune complexes that helps moDC to stimulate IFN-γ production by PBMC, and promote CD8 T cell expansion: particularly in a subset of donors that do not respond well to virus alone.

The CD8 T cell proliferation promoted by the modified Fc is antigen-specific

To understand if the CD8 T cell response was antigen-specific, we studied the response of donor LCY02; this donor has the haplotype HLA-A24 and HLA-A2, and exhibited a 2.7-fold increase in the percentage of CD8 T cells in moDC: $CD14^-$ PBMC co-cultures in response to Fc-modified immune complexes (Supplemental Fig. 3). We re-stimulated the expanded CD8 T cells in the co-cultures using autologous moDC that had been treated with TNF-α and pulsed with peptides representing an adenovirus epitope for HLA-A24 haplotype (TYFSLNNKF), HLA-A2 haplotype (YVLFEVFDVV), and, as negative controls, a scrambled peptide (LAVFEDYVAF) or a HIV epitope (SLYNTVATL). We also tested the antigen-specific response using a library of 15-mer peptides representing the hexon protein of adenovirus or an irrelevant protein (Human NY-ESO-1 protein).

After 16 h of re-stimulation, a marked and specific IFN-γ response to the HLA-A24-restricted-adenovirus epitope (TYFSLNNKF) (Fig. 6A), and to the peptide library generated from the adenovirus-hexon protein (Fig. 6B) was seen suggesting that the immune response was specific to adenovirus.

In the same experiment, we labelled the $CD14^-$ PBMC with CFSE to enable us to track which cell populations had proliferated. After 11 days of co-culture with moDCs that were treated with the Fc-modified immune complex, about 43% of the CD8 T cells, and 21% of the CD4 T cells had proliferated (Fig. 6C). Re-stimulating the co-culture with moDC that were pulsed with HLA-A24 adenovirus peptide cause 6.57% of the proliferated CD8 T cells to produce IFN-γ, while the response to the HLA-A2 adenovirus peptide and the negative controls remained at around 1% (Fig. 6C). Re-stimulating the co-culture with moDC that were pulsed with the Ad5 hexon peptide library (Fig. 6C) also caused 3.46% of the proliferated CD8 T cells and 4.73% of the proliferated CD4 T cells to produce IFN-γ, while the IFN-γ producing CD4 and CD8 T cells remained at around 1% of the proliferated cells for the negative control (NY-ESO-1 peptide library).

Since the single peptide experiments used 9-mer peptides, we did not expect the CD4 T cells to be re-stimulated; however, there was a small increase in the frequency of IFN-γ-producing CD4 T cells in co-cultures where moDC were treated with the HLA-A24 peptide relative to negative controls. This could be a bystander effect from the production of cytokines by the CD8 T cells that were truly responding to the 9-mer peptide.

To remove the bystander effect of cytokines which could be released during re-stimulation, we repeated the experiment and added Brefeldin A to stop cytokine release right at the start of re-stimulation. We found that only CD8 but not CD4 T cell show antigen-specific re-stimulation (Fig. 6D). Controls using CD3/CD28 agonist confirm that the CD4 T cells are capable of responding to stimulation and are not intoxicated by the overnight treatment with Brefeldin A. Thus, our results indicate that the CD8 T cell proliferation stimulated by Fc-modified immune complexes is antigen-specific.

Discussion

We engineered the human IgG1 Fc region to increase its affinity for TRIM21 by 100-fold, and showed that the resulting antibodies directs viral antigens effectively into the cross-presentation pathway leading to the stimulation of antigen-specific CD8 T cells. This process is mediated by cross-presentation and not the classical MHCI presentation of intracellularly expressed viral proteins because most of the endocytosed viruses are neutralized and therefore viral genes are not expressed. Importantly, the cross-presentation process is remarkably enhanced by Fc-modification for increased TRIM binding, while the host-protective mechanism of ADIN is retained.

Both the Fcγ receptors (FcγRs) and the neonatal Fc receptor (FcRn) have been shown to facilitate cross-presentation (16, 25). However there is yet to be a study to demonstrate that TRIM21 also accesses this pathway. The involvement of TRIM21 with the proteasome has led to speculation that it regulates antigen-processing (signal 1) in DC, but we found that TRIM21 ligation by Fc-modified immune complexes also induces expression of co-stimulatory molecules (signal 2), and pro-inflammatory cytokine/chemokine release (signal 3) by moDC. For cross-priming of CD8 T cells, all 3-signals are required. DC-targeted vaccine strategies often rely on a cocktail of four cytokines, IL1β, IL-6, TNF-α and Prostaglandin E_2 (25–27), to provide signals 2 and 3 to DC: our Fc-modified immune complexes could potentially provide all three signals. This removes the need to optimize the timing and dosages of the different components and also overcomes the challenge of ensuring all four components reaches the same DC at the right timing *in vivo*: in other words, the modified Fc would render *in vivo* application of DC-targeted vaccines more feasible.

MoDCs treated with Fc-modified immune complexes consistently upregulate maturation marker expression, but only a sub-population of donors exhibit markedly increased CD8 T cell expansion. These differences are likely to reflect differences in previous Ad5-exposure of the donors, and will be investigated further in future studies. Donors with prior exposure to adenovirus could have memory T cells that are capable of responding to viral antigens in the absence of co-stimulatory molecule expression by DC. In this case, we would expect the moDC treated with virus alone to outperform those treated with immune complexes since there is an increased expression of viral proteins in the absence of ADIN (28). Having a larger population of moDC infected and producing viral proteins, albeit without upregulating their co-stimulatory markers, will stimulate memory T cells in the subpopulation of donors who have them. Importantly, prophylactic vaccines need to stimulate naïve T cells rather than memory T cells, since they are meant to protect individuals with no prior exposure to the virus; while therapeutic vaccines need to revive a virally-damped immune response,

which means that the DC might need a “boost” to upregulate their stimulatory status again. In both cases, the ability of the modified Fc to enhance moDC-cross-priming of CD8 T cells should address these issues.

Here we used Ad5 immune complexes to demonstrate the potential of Fc-modified immune complexes to induce and enhance CD8 T cell responses to viral antigen, but these findings are of relevance to other pathogens and diseases. Ad5-immune complexes reach TRIM21 in the cytosol of moDC because adenoviruses releases Protein VI which lyses the endosome (29, 30); to extend the scope of the modified Fc to other clinically-relevant targets, future work should explore the possibilities of using the modified Fc on DC-targeting antibodies such as anti-DEC205 and anti-CLEC9A. For example, antibody-antigen fusion proteins anti-DEC205-NY-ESO-1(31) and anti-DEC-HIV gag (32) have been shown to stimulate anti-cancer and anti-HIV CD8 T cell responses. If these antibody-antigen fusion proteins can reach TRIM21 intact, the scope of application for the modified Fc will be tremendous. In a different mode of application, antibodies can be designed to target antigens of interest to form immune complexes that are taken into the DC via the Fc receptor. Fc receptor-mediated antigen internalization in DC are known to be channeled to a special transport pathway which allows the antigen efficient access to the cytosol (33). HRP-anti-HRP immune complexes were detectable by HRP substrate (DAB) and anti-rabbit IgG Fabs suggesting that both antigen and antibody remains mostly intact in the cytosol (34). Given that an Fc receptor (TRIM21) and an antigen-processing enzyme (proteasome) both exist in the cytosol, it is possible that immune complexes have privileged access to deliver themselves and their cargo “completely intact” to the cytosol.

In summary, we have identified a readily-adaptable method of Fc modification for targeting endocytosed antigen in immune complexes to the MHCI cross-presentation pathway via TRIM21, and have shown its potential to provide all the signals necessary for the stimulation of a potent CD8 T cell and cytokine response to specific antigen.

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The authors declare no competing interests.

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Figures

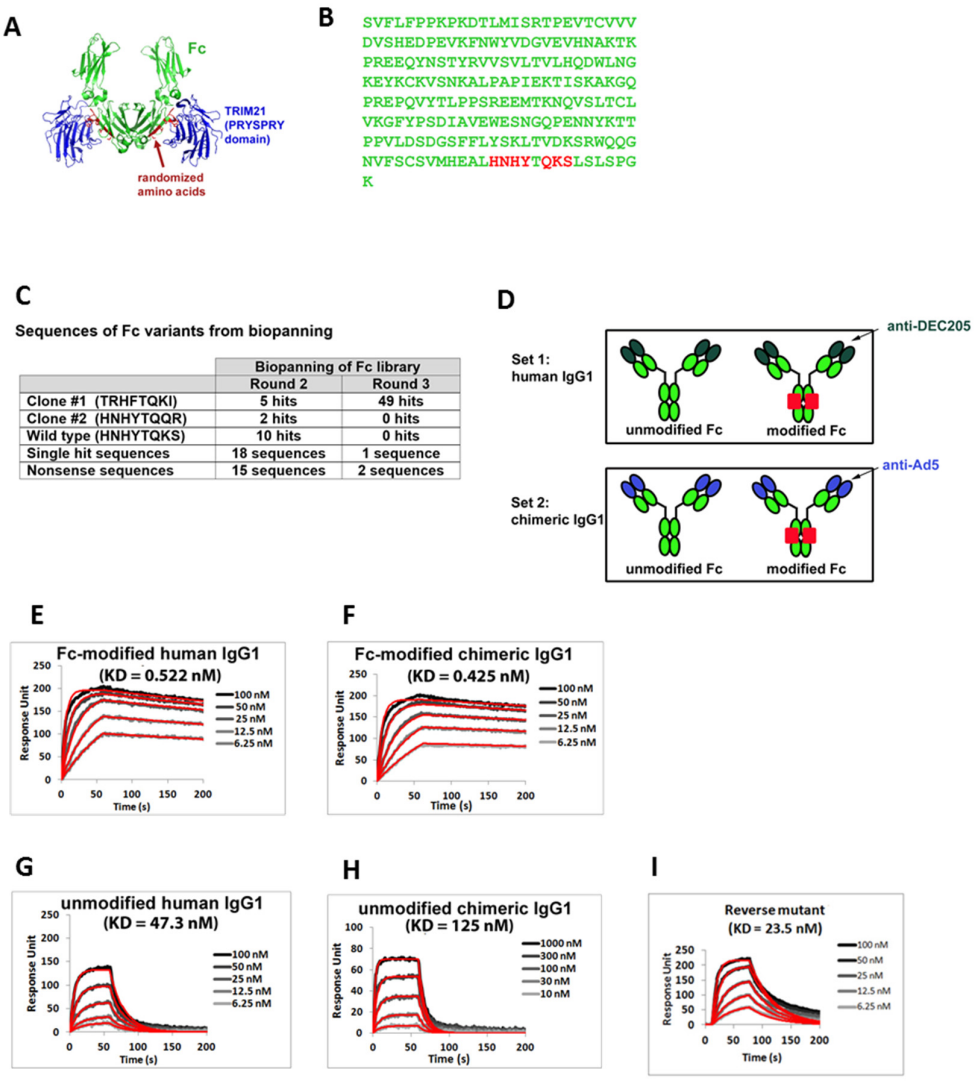
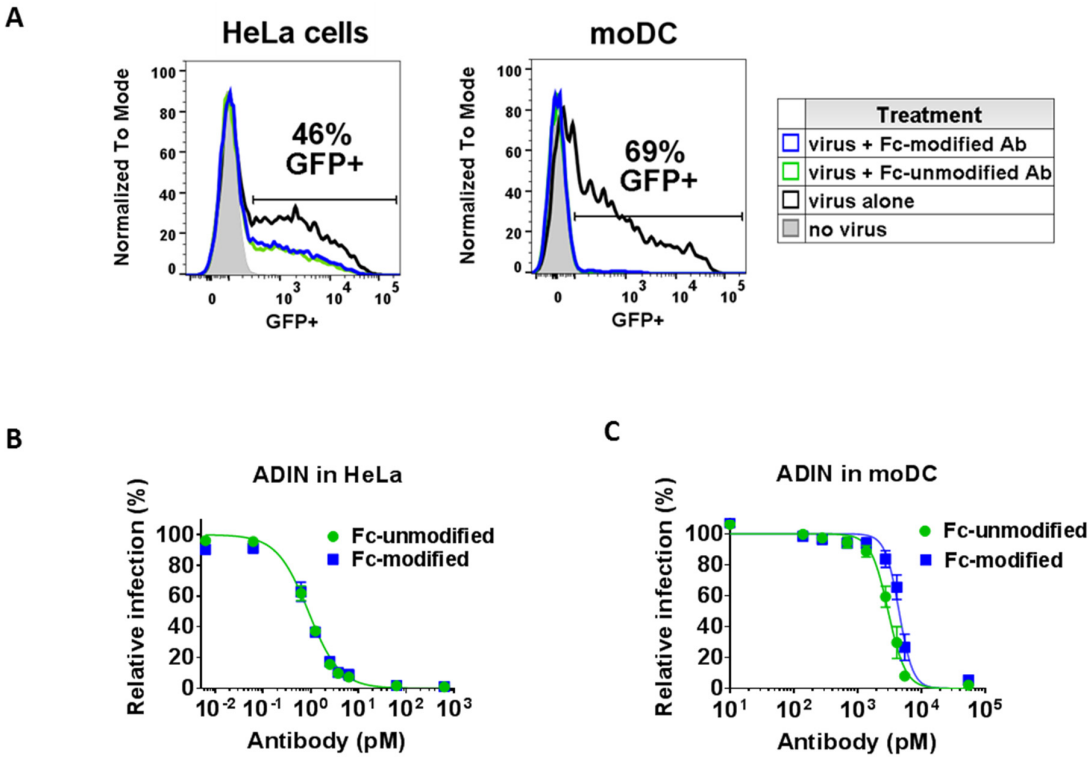


FIGURE 1. A modified Fc that exhibits increased affinity for TRIM21. **(A)** Crystal structure from Protein Data Base (PDB:2IWG) showing TRIM21 PRYSPRY domain (blue) in complex with Fc (green). The amino acids selected for randomization are in red. **(B)** The protein sequence of Fc is shown. The amino acids selected for randomization are in red. **(C)** Frequency of the different clones identified after Round 2 and Round 3 of biopanning. **(D)** Construction of two sets of antibodies: anti-DEC205 human IgG1 and anti-Ad5 chimeric IgG1. For each set, the Fc regions modified (red squares) or unmodified. **(E-I)** Binding and dissociation of TRIM21 to IgG1 detected using surface plasmon resonance. The grey to black lines are the experimental data. The red lines are curve fittings using Langmuir 1:1 stoichiometry. The IgG1s are **(E)** Fc-modified human IgG1, **(F)** Fc-modified chimeric IgG1, **(G)** unmodified human IgG1, **(H)** unmodified chimeric IgG1 and **(I)** the reverse mutant which is the same as the Fc-modified chimeric IgG1 but with the spurious mutation T256P corrected back to threonine.



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676 **FIGURE 2.** Increasing Fc affinity for TRIM21 does not affect ADIN. **(A)** FACS plot showing
677 HeLa cells and moDC at 48 h after treatment with no virus, virus alone or virus with
678 antibodies. For HeLa cells an MOI of 1.2 is used and an antibody concentration of 1.25 pM is
679 used. For moDC, an MOI of 400 is used and an antibody concentration of 55 nM is used.
680 **(B and C)** ADIN mediated by Fc-modified and unmodified antibodies at different
681 concentrations. The graphs shows the mean relative infection and the SEM of three
682 different donors. **(B)** HeLa cells were infected with adenovirus at an MOI of 1.2. **(C)**
683 moDCs were infected with adenovirus an MOI of 400.

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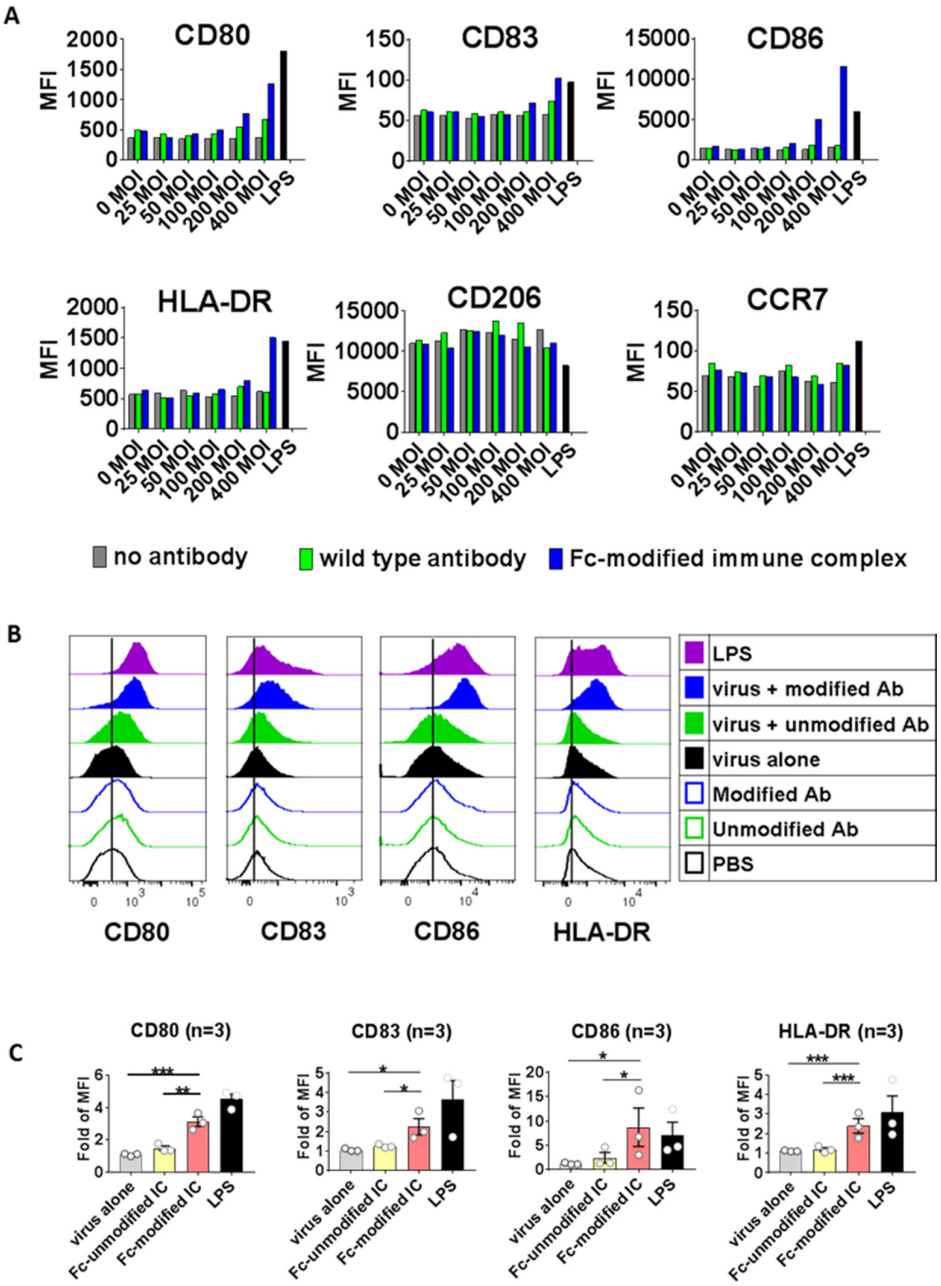

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687 **FIGURE 3.** Increasing Fc affinity for TRIM21 promotes moDC maturation markers. **(A)**
688 moDC are treated with Ad5 alone, Fc-unmodified immune complexes or Fc-modified
689 immune complexes at an MOI of 0, 25, 50, 100, 200 and 400. The immune complexes were
690 form by pre-incubating the virus and antibody at 5 x 10^8 virus and 20 µg antibody per ml.
691 Thereafter the amount of immune complex is added based on the MOI of the virus. LPS
692 (1ug/ml) is a positive control. Graph shows the result for one representative donor (Donor
693 22). **(B)** Histogram showing the MFI of different maturation markers when moDC is treated
694 at an MOI of 400 MOI. The concentration of the antibody alone or in the immune complexes
695 is 55 nM. Histogram shows results for one representative donor (Donor 22). **(C)** Fold
696 increase in MFI relative to treatment with virus for 3 donors. Each donor is represented by a
697 circle. Error bar shows the SEM. Statistical analysis are done using ordinary one-way
698 ANOVA. The adjusted P values are represented by * for $p<0.05$, ** for $p<0.01$ and *** for
699 $p<0.001$.

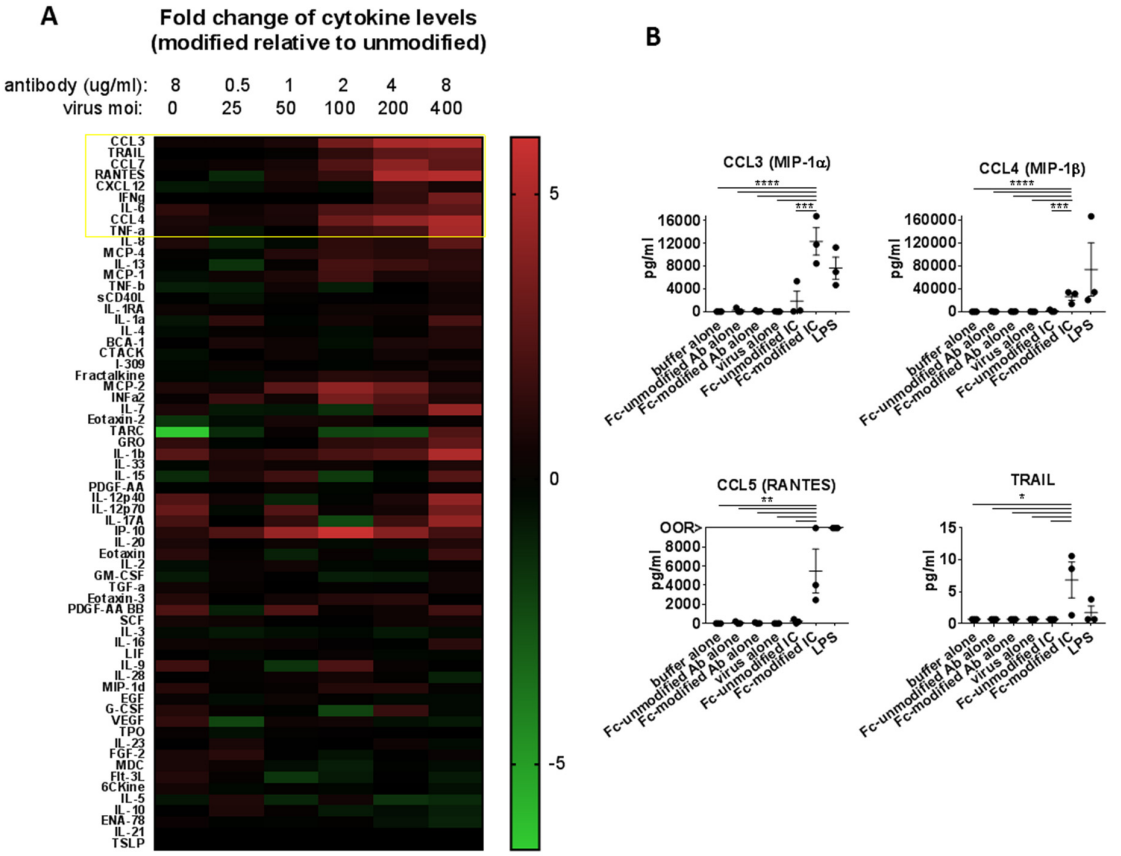

FIGURE 4. Increasing Fc affinity for TRIM21 promotes moDC production of Th1-associated chemokines. **(A)** Fold change in cytokine and chemokine concentration relative to treatment with unmodified immune complex. The heat map shows the cytokines and chemokines assayed using human Milliplex MAP kits immunology panel 1 and 2, which includes a total of 64 targets. The color scale is $\log_2$ and ranges from red to green color; with red representing 2^5-fold upregulated and green representing 2^5-fold downregulated expression in Fc-modified immune complex when compared to Fc-unmodified immune complex. The cytokines and chemokines are ranked by the Spearman rho correlation (for fold change versus dosages). The top nine cytokines and chemokines (yellow box) show a Spearman rho of >0.3 at $p<0.05$. **(B)** Concentration of the four chemokines and cytokines, CCL3, CCL4, CCL5 and TRAIL. Each donor is represented by a circle. IC: immune complexes. Graph shows mean and SEM of three donors. Statistical analysis are done using ordinary one-way ANOVA. For statistical analysis, the adjusted p-values are represented by **** for $p<0.0001$, *** for $p<0.001$ ** for $p<0.01$ and * for $p<0.05$.

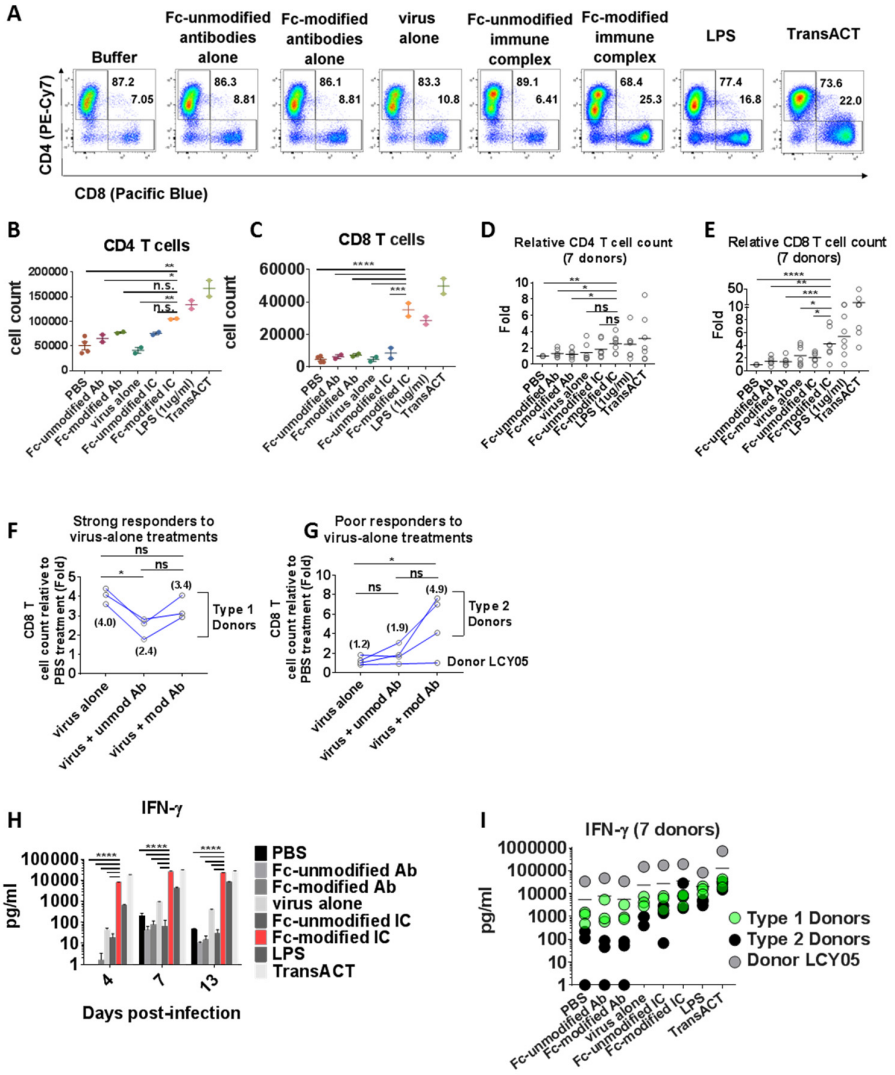

FIGURE 5. Increasing Fc affinity for TRIM21 helps moDC promote CD8 T cell expansion. **(A)** Flow cytometry analysis of moDC: $CD14^-$ co-culture at 13 days post infection of moDC with virus alone, immune complexes or controls. Cells are gated by singlets/live/$CD3^+CD56^-$. The numbers in the plots show the percentage of CD4 T cells and CD8 T cells in the total T cells. **(B and C)** Number of **(B)** CD4 T cells and **(C)** CD8 T cells in the co-culture after 13 days. Each dot is a replicate of the experiment on one donor (Donor 33). Error bar shows the SEM of the replicates. **(D and E)** Fold change in **(D)** CD4 T cell count and **(E)** CD8 T cell count relative to PBS treatments. Each dot represents one donor and the line represents the mean of 7 donors. **(F and G)** Fold change in CD8 T cell count relative to PBS treatments for **(F)** Type 1 donors and **(G)** Type 2 donors. Numbers in brackets indicates the mean fold change. **(H)** ELISA analysis at 4, 7 and 13 days post infection by virus, immune complexes or controls of a Type 2 donor. The graph show the mean and SEM of triplicates for one representative donor (LCY10). **(I)** Analysis of the level of IFN-γ for the 7 donors at 7 dpi with with Type 1 and Type 2 donors highlighted in green and black respectively. Each dot represents one donor. For all statistical analysis, ordinary ANOVA was performed. LPS and TransACT are omitted from statistical analysis. For statistical results, **** represents $p<0.0001$, *** represents $p<0.001$ ** represents $p<0.01$ and * represents $p<0.05$ and ns is not significant with $p>0.05$.

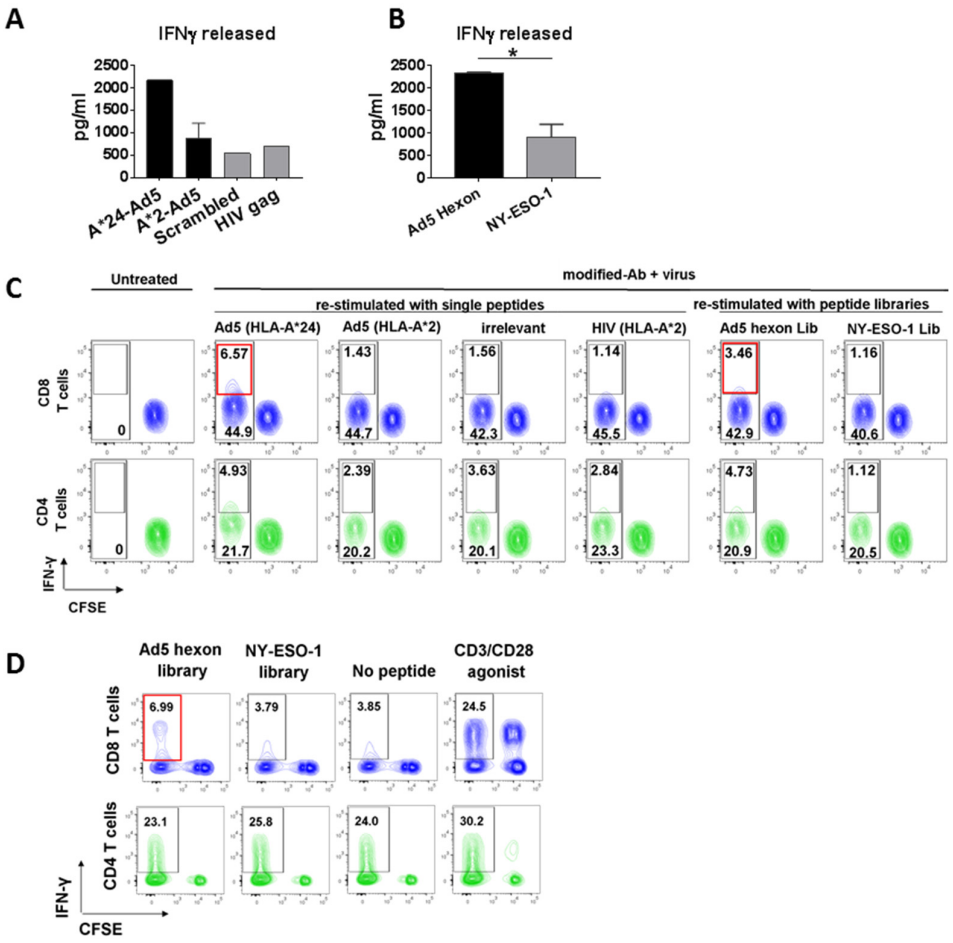

FIGURE 6. CD8 T cell proliferation promoted by the modified Fc is antigen-specific. **(A and B)** ELISA detecting the level of IFN-γ in the supernatant of moDC: CD14$^-$ PBMCs co-cultures that were re-stimulated for 16 h by autologous moDC pulsed with peptides. Results show the means and SEM of duplicates (for A*2-Ad5, Ad5 Hexon and NY-ESO-1 treatments) or of a single well (for A*24-Ad5, Scrambled and HIV gag treatments) of donor LCY02. **(A)** A*24-Ad5 and A*2-Ad5 are peptides from Adenovirus that is presented by HLA-A24 and HLA-A2 haplotypes respectively. Scrambled and HIV gag are negative controls. **(B)** Ad5 Hexon is a peptide library of 15-mer peptides from the hexon protein of Adenovirus (Ad5 hexon), NY-ESO-1 is a peptide library of 15-mer peptides from the human protein NY-ESO-1. **(C)** Flow cytometry analysis of donor LCY02's CD4 and CD8 T cells from CD14$^-$ PBMC co-cultures with autologous moDC that were untreated or treated with modified Fc immune complex for 11 days. The co-culture was re-stimulated by moDC pulsed with different peptides for 16 h. Thereafter cells were treated for 5 h with Brefeldin A and then stained for flow cytometry analysis. Numbers at the bottom show percentages of CFSE-diluted cells in the CD8 or CD4 T cells populations. Numbers at the top show percentages of IFN-γ high cells in the population of CFSE-diluted CD8 or CD4 T cells. In red boxes are responses that are specific to re-stimulation by the antigen. **(D)** Flow cytometry analysis of donor LCY10's CD4 and CD8 T cells from CD14$^-$ PBMC co-cultures with autologous moDC that were treated with modified Fc immune complex for 11 days and then re-stimulated for 16 h by moDC pulsed with different peptides, while in the presence of Brefeldin A. Numbers at the top show percentage of IFN-γ high cells in a population of CFSE-diluted CD8 or CD4 T cells. The red box show responses that are specific to re-stimulation by the antigen.