An Activating Immunoreceptor Complex Formed by NKG2D and DAP10

Jun Wu,[1] Yaoli Song,[1] Alexander B. H. Bakker,[1] Stefan Bauer,[2] Thomas Spies,[2] Lewis L. Lanier,[1]* Joseph H. Phillips[1]

Many immune receptors are composed of separate ligand-binding and signal-transducing subunits. In natural killer (NK) and T cells, DAP10 was identified as a cell surface adaptor protein in an activating receptor complex with NKG2D, a receptor for the stress-inducible and tumor-associated major histocompatibility complex molecule MICA. Within the DAP10 cytoplasmic domain, an Src homology 2 (SH2) domain–binding site was capable of recruiting the p85 subunit of the phosphatidylinositol 3-kinase (PI 3-kinase), providing for NKG2D-dependent signal transduction. Thus, NKG2D-DAP10 receptor complexes may activate NK and T cell responses against MICA-bearing tumors.

The ability of NK cells to kill tumors and virus-infected cells and to produce cytokines is regulated by a balance between activating and inhibitory receptors. Inhibition is mediated by receptors for major histocompatibility complex (MHC) class I, including Ly49 and the killer cell immunoglobulin-like receptor (KIR) (*1*). These receptors have immunoreceptor tyrosine-based inhibition motifs in their cytoplasmic domains that recruit cytoplasmic tyrosine phosphatases, resulting in inactivation of NK cell function (*2*). However, certain receptors within the KIR and Ly49 families activate NK cells (*3*). These receptors lack signaling motifs but associate with DAP12, a CD3-like protein with an immunoreceptor tyrosine-based activation motif (*4*). Engagement of such receptor complexes triggers a signaling cascade similar to that initiated by the T cell receptor (*4*).

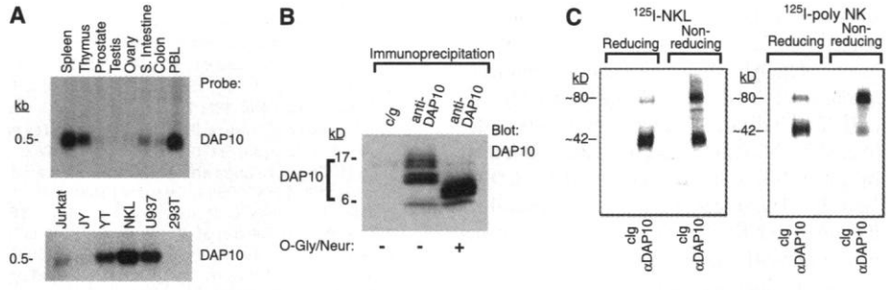
A sequence with ~20% amino acid homology to DAP12 was identified as a human expressed sequence tag. This cDNA encodes DAP10, a type I membrane protein of 93 amino acids (Fig. 1). Its transmembrane (TM) contains a negatively charged residue that is conserved in DAP12 and in the CD3 subunits. The short cytoplasmic region of DAP10 has a Tyr-X-X-Met (YXXM) motif, a potential SH2 domain–binding site for the p85 subunit of the PI 3-kinase (*5*), suggesting a role for DAP10 as a signaling adaptor. Southern (DNA) blot analysis revealed a restriction enzyme digestion pattern predicted by the genomic sequence, consistent with a single *DAP10* gene (*6*). The human *DAP10* and *DAP12* genes are on human chromosome 19q13.1 in opposite transcriptional orientation, separated by only ~130 base pairs (bp) (*6*). Abundant ~500-bp DAP10 transcripts were detected in human peripheral blood leukocytes, spleen, thymus, NK cells, α/β− and γ/δ−T cell receptor$^+$ T cells, and U937 (myeloid cell), but not substantially in other tissues or JY (B lymphoblastoid cell), 293T (epithelial cell), or primary fibroblasts (Fig. 2A) (*6*). Analysis by the reverse transcription polymerase chain reaction (RT-PCR) indicated the presence of *DAP10* mRNA in CD4$^+$ and CD8$^+$ T cell clones and in monocytes, granulocytes, and dendritic cells (*6, 7*). Thus, DAP10 is predominantly expressed in hematopoietic cells.

Protein immunoblot analysis of the NK cell line NKL, using an affinity-purified antibody to DAP10 (anti-DAP10), revealed multiple bands migrating slower than the predicted molecular mass of 10 kD for DAP10, primarily as a result of O-linked glycosylation (Fig. 2B). The multiple bands observed after treatment with *O*-glycanase may be due to incomplete saccharide removal, other post-translational modifications, or alternative splicing. To examine whether DAP10 associates with other membrane receptors, we lysed a ^{125}I-labeled polyclonal NK line and NKL in 1% digitonin to preserve multisubunit receptor complexes. Although DAP10 did not label with ^{125}I, anti-DAP10 coprecipitated a ^{125}I-labeled glycoprotein migrating at ~42 kD under reducing conditions and at ~42 and ~80 kD under nonreducing conditions (Fig. 2C). Removal of N-linked sugars revealed a ~28-kD polypeptide (Fig. 2D). Similar ^{125}I-

MIHLGHILFLLLLPVAAAQTTPGERSSLPAFYPGTSGSCSGCGSLSLP
Leader — Extracellular Domain

LLAGLVAADAVASLLIVGAVFLCARPRRSPAQEDGKVYINMPGRG
Transmembrane — Cytoplasmic Domain

Fig. 1. Human DAP10. The extracellular cysteine residues, the transmembrane charged residue, and a cytoplasmic signaling motif are bold. Human *DAP10* cDNA (AF122904) and a splice variant (AF072844) were identified, as were mouse *DAP10* cDNA (AF072846) and a splice variant (AF122905). The genomic organization of human *DAP10* (AF072845) was deduced from a fragment of human chromosome 19q13.1 (AD0008333). All numbers in parentheses are GenBank accession numbers.



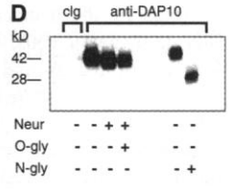


Fig. 2. DAP10 RNA and protein. (**A**) Northern blot analysis of DAP10 in human tissues and a T leukemia cell (Jurkat), a B lymphoblastoid cell (JY), an NK leukemia cell (YT), an NK cell line (NKL), a myeloid cell (U937), and an epithelial cell (293T) (*14*). Blots were stripped and rehybridized with an actin probe to confirm that all lanes were equally loaded (not shown). The small amounts of DAP10 detected in the nonhematopoietic organs may be contributed by tissue macrophages. (**B**) Lysates prepared from NKL were immunoprecipitated with a control immunoglobulin (clg) or affinity-purified anti-DAP10. Immune complexes were either untreated or treated with neuraminidase and *O*-glycosidase (O-Gly/Neur). Samples were analyzed by protein immunoblot using affinity-purified anti-DAP10 (*15*). (**C** and **D**) NKL or a polyclonal NK cell line were labeled with ^{125}I, lysed in 1% digitonin, and immunoprecipitated with clg or affinity-purified anti-DAP10. Samples were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE) under reducing or nonreducing conditions (C) or were treated with neuraminidase (Neur), O-glycosidase (O-gly), or N-glycosidase F (N-gly), separately and in combination, and analyzed under reducing conditions (D) (*16*).

[1]DNAX Research Institute, 901 California Avenue, Palo Alto, CA 94304, USA. [2]Fred Hutchinson Cancer Research Center, Clinical Research Division, Seattle, WA 98109, USA.

*To whom correspondence should be addressed. E-mail: lanier@dnax.org

labeled DAP10-associated proteins were detected in several NK and T cell clones (*7*).

The precedent for interactions between proteins of multisubunit receptor complexes through oppositely charged amino acids in their transmembranes suggested that DAP10 may pair with another ligand-binding subunit. One candidate was NKG2D, a C-type lectin encoded by a gene in the "NK complex" on human chromosome 12p12-p13 (*8*). The mouse pre-B cell line Ba/F3 was transfected with an NH_2-terminal Flag-tagged human DAP10 cDNA (Flag-DAP10), either alone or together with human NKG2D. Stable transfectants were stained using Flag monoclonal antibody (mAb) to visualize DAP10 and NKG2D mAb to detect NKG2D (*9*). Cotransfection of Flag-DAP10 and NKG2D resulted in surface expression of both proteins, whereas alone they were minimally expressed (Fig. 3A). Radioiodination and coimmunoprecipitation confirmed that DAP10 and NKG2D form a stable complex on the double transfectant (*7*). As with other receptor complexes, charged residues in the TM of NKG2D and DAP10 were critical for complex formation because mutation of these amino acids allowed surface expression and abolished the stable association between DAP10 and NKG2D (Fig. 3B). Small amounts of Flag-DAP10 were on the surface of Ba/F3 cells expressing the NKG2D TM mutant. However, we were unable to coimmunoprecipitate the NKG2D TM mutant with DAP10 (*7*). Residues in the TM other than Arg may affect the pairing of NKG2D with DAP10.

To establish that NKG2D is the physiological partner for DAP10, we immunoprecipitated ^{125}I-labeled NKL and a polyclonal NK line with an affinity-purified rabbit antibody specific for the cytoplasmic domain of human NKG2D or with a human NKG2D mAb. The size of the DAP10-associated glycoprotein was identical to that of NKG2D (Fig. 3C). Moreover, NKG2D was reimmunoprecipitated from the eluate of the dissociated DAP10 complex (Fig. 3C). DAP10 could not pair with KIR2DS2 or CD94/NKG2C, two receptors associated with DAP12 (*4*), and DAP12 did not associate with NKG2D; these results confirmed the specificity of the interaction (*7*). The extracellular domains of DAP10 and DAP12 contain cysteines that form disulfide-bonded homodimers; however, heterodimers were not observed (*7*). NKG2D, despite its name, has limited homology with NKG2A, -C, and -E (*8*) and does not form heterodimers with CD94 (*7*).

NKG2D is an activating receptor (*6*) that initiates NK and T cell–mediated cytotoxicity against transfectants and tumors expressing its ligands, MICA and MICB (*9*). However, NKG2D lacks signaling elements in its cytoplasmic domain. A potential activation motif in the cytoplasmic domain of DAP10 is the YXXM sequence, a predicted binding site for the SH2 domain of the p85 subunit of PI 3-kinase (*5*). A tyrosine-phosphorylated peptide corresponding to the DAP10 cytoplasmic domain specifically bound to p85 (Fig. 4A). Treatment of NKL and NKG2D-Flag-DAP10$^+$ Ba/F3 with the phosphatase inhibitor pervanadate resulted in DAP10 tyrosine phosphorylation and enhanced association of p85 with the NKG2D-DAP10 complex (Fig. 4B). This suggested that DAP10 functions as a signal transducer leading to PI 3-kinase activation.

CD28 is a well-characterized receptor with a cytoplasmic YXXM motif that activates PI 3-kinase (*10*). On T cells CD28 is a costimulatory molecule, whereas on NK cells CD28 initiates cytotoxicity against target cells expressing CD28 ligands (CD80 or CD86) (*11*). Unlike CD28, DAP10 has a small extracellular domain and is unlikely to mediate ligand binding, whereas NKG2D directly binds MICA (*7, 9*). Having separate components of a multisubunit receptor responsible for ligand binding and signaling may permit DAP10 to function as an adaptor molecule for other receptors (for example, in myeloid cells expressing DAP10 but lacking NKG2D). The finding that the NKG2D-DAP10 complex is a receptor for the nonclassical MHC class I molecule MICA (*9*) and evidence that these molecules are stress-inducible and broadly expressed in epithelial tumors (*12*) suggest that the activating NKG2D-DAP10 complex may be involved in innate immune surveillance.

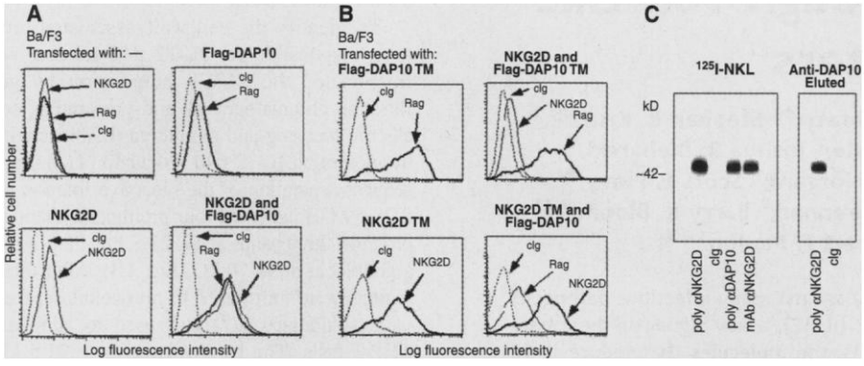


Fig. 3. An NKG2D-DAP10 receptor complex. (**A** and **B**) Ba/F3 cells stably expressing the indicated receptors were stained with clg, Flag mAb M2, or NKG2D mAb 5C6 [or 1D11 mAb (*7*)] and analyzed by flow cytometry. Flag-DAP10 TM and NKG2D TM contain point mutants in the transmembranes in which the charged residues were substituted with Ala and Leu, respectively (*17*). (**C**) ^{125}I-labeled NKL cells were lysed in 1% digitonin and immunoprecipitated with clg, affinity-purified anti-NKG2D, affinity-purified anti-DAP10, or NKG2D mAb 5C6. DAP10-associated proteins were eluted with 50 mM diethylamine (pH 12) and reimmunoprecipitated with either clg or affinity-purified anti-NKG2D. Samples were analyzed by SDS-PAGE (reducing condition).

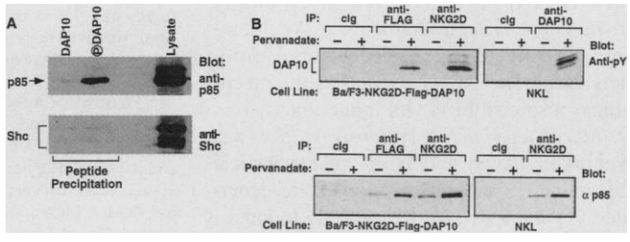


Fig. 4. DAP10 recruits PI 3-kinase. (**A**) NKL lysates were incubated with a biotinylated unphosphorylated (DAP10) or phosphorylated DAP10 peptide (biotin-PAQE-DGKVY* INMPGRG; Y* indicates the phosphotyrosine residue) (P-DAP10) and precipitated with avidin-agarose. Samples were analyzed by protein immunoblot using anti-p85 (*18*). (**B**) NKG2D-DAP10$^+$ Ba/F3 cells or NKL were stimulated with pervanadate, lysed, and immunoprecipitated with clg, Flag mAb M2, or NKG2D mAb 5C6. Samples were analyzed by protein immunoblot using HRP-conjugated phosphotyrosine mAb 4G10 or anti-p85 (*19*). Blots were stripped and rehybridized with anti-Shc (specificity control).

References and Notes

1. L. L. Lanier, *Annu. Rev. Immunol.* **16**, 359 (1998).
2. D. N. Burshtyn *et al.*, *Immunity* **4**, 77 (1996); A. Fry, L. L. Lanier, A. Weiss, *J. Exp. Med.* **184**, 295 (1996); K. S. Campbell, M. Dessing, M. Lopez-Botet, M. Cella, M. Colonna, *ibid.*, p. 93.
3. L. H. Mason *et al.*, *J. Exp. Med.* **184**, 2119 (1996); R. Biassoni *et al.*, *ibid.* **183**, 645 (1996).
4. L. L. Lanier, B. C. Corliss, J. Wu, C. Leong, J. H. Phillips, *Nature* **391**, 703 (1998).
5. Z. Songyang *et al.*, *Cell* **72**, 767 (1996).
6. See *Science* Online (www.sciencemag.org/feature/data/1040915.shl) for more data on the genomic organization of the human *DAP10* and *DAP12* genes, DAP10 Southern blot analysis, DAP10 expression in various hematopoietic cells, and NKG2D activity.
7. J. Wu, T. Spies, J. H. Phillips, L. L. Lanier, unpublished data.
8. J. P. Houchins, T. Yabe, C. McSherry, F. H. Bach, *J. Exp. Med.* **173**, 1017 (1991).

9. S. Bauer *et al.*, *Science* **285**, 727 (1999).
10. F. Pages *et al.*, *Nature* **369**, 327 (1994); K. E. Truitt, C. M. Hicks, J. B. Imboden, *J. Exp. Med.* **179**, 1071 (1994).
11. M. Azuma *et al.*, *J. Immunol.* **149**, 1114 (1992); L. L. Lanier *et al.*, *ibid.* **154**, 97 (1995); B. J. Chambers, M. Salcedo, H.-G. Ljunggren, *Immunity* **5**, 311 (1996).
12. V. Groh *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 12445 (1996); V. Groh, A. Steinle, S. Bauer, T. Spies, *Science* **279**, 1737 (1998); V. Groh, R. Rhinehart, H. Secrist, K. Grabstein, T. Spies, *Proc. Natl. Acad. Sci. U.S.A.* **96**, 6879 (1999).
13. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
14. Northern and Southern analysis using human DNA and RNA (Clontech) were performed as described (*4*).
15. Rabbits were immunized with peptides corresponding to the cytoplasmic domain of either human DAP10 (PAQEDGKVYINMPGRG) (Anaspec, San Jose, CA) or human NKG2D (KKSDFSTRWQKQR) (Research Genetics, Birmingham, AL) (*13*). Antisera were affinity-purified with the immunizing peptide. Specificity was determined by testing the affinity-purified antibodies by immunoprecipitation and protein immunoblot analysis on Ba/F3 cells transfected with human DAP10 and NKG2D (or both), compared with preimmune sera or control antibodies.
16. Cells were labeled with ^{125}I and solubilized [1% digitonin, 0.12% Triton X-100, 150 mM NaCl, 20 mM triethanolamine, 0.01% NaN_3 (pH 7.8), and protease inhibitors] (*4*). Lysates were incubated (2 hours, 4°C) with Pansorbin (Calbiochem) coated with clg, affinity-purified anti-DAP10, and affinity-purified anti-NKG2D. For immunoprecipitation with mouse mAb, Pansorbin was precoated with rabbit antibody to mouse Ig (Sigma) and mouse NKG2D mAb 5C6, anti-Flag mAb M2 (Sigma), or clg. Immunoprecipitates were washed in buffer [5 mM CHAPS, 50 mM tris, 150 mM NaCl (pH 8.0), and protease inhibitors] (*4*).
17. A cDNA containing the human CD8 leader segment, followed by the Flag epitope (DYKDDDDK), and joined to the extracellular, transmembrane, and cytoplasmic segments of human DAP10, was subcloned into the pMX-neo retroviral vector (*4*, *13*). Human NKG2D cDNA (*8*) was subcloned into pMX-puro. The D-A transmembrane Flag-DAP10 mutant cDNA with an A (GCC) substituted for D (GAT) and the R-L transmembrane NKG2D mutant cDNA with an L (CTT) substituted for R (CGT) were generated by PCR mutagenesis. Retroviruses were generated using the Phoenix packaging cell (*4*). Ba/F3 cells were infected and drug-selected, and transfectants were isolated by flow cytometry (*4*).
18. Phosphorylated or unphosphorylated biotinylated peptides of the cytoplasmic domain of DAP10 (PAQEDGKVYINMPGRG) were incubated with lysates from NKL, precipitated with avidin-agarose, and washed [1% NP-40, 50 mM tris, 150 mM NaCl (pH 7.8), and protease inhibitors] (*4*, *13*). Immunoprecipitates were analyzed by protein immunoblot using either anti-p85 or anti-Shc (specificity control) (UBI, Lake Placid, NY).
19. NKL and NKG2D-Flag-DAP10$^+$ Ba/F3 cells were incubated with 100 mM pervanadate and lysed (0.875% Brij 97, 0.125% NP-40, 150 mM NaCl, 10 mM tris, and protease and phosphatase inhibitors) (*4*). Antigens precipitated with clg, Flag mAb M2, or NKG2D mAb 5C6 were analyzed by protein immunoblot using horseradish peroxidase (HRP)–conjugated phosphotyrosine mAb 4G10 (UBI) or antiserum to p85 (UBI).
20. We thank M. Robertson, J. P. Houchins, C. Turck, G. Nolan, and T. McClanahan for reagents; J. Katheiser, G. Burget, and M. Andonian for graphics; J. Cupp, E. Callas, and D. Polakoff for flow cytometry; and D. Gorman and D. Liggett for sequencing. DNAX is supported by Schering Plough. S.B. and T.S. are supported by NIH grant AI30581.

14 April 1999; accepted 24 June 1999

Host Defense Mechanisms Triggered by Microbial Lipoproteins Through Toll-Like Receptors

Hans D. Brightbill,[1]* Daniel H. Libraty,[4]* Stephan R. Krutzik,[1] Ruey-Bing Yang,[6] John T. Belisle,[7] Joshua R. Bleharski,[1] Michael Maitland,[8] Michael V. Norgard,[9] Scott E. Plevy,[10] Stephen T. Smale,[1,2,5] Patrick J. Brennan,[7] Barry R. Bloom,[8,11] Paul J. Godowski,[6] Robert L. Modlin[1,2,3]†

The generation of cell-mediated immunity against many infectious pathogens involves the production of interleukin-12 (IL-12), a key signal of the innate immune system. Yet, for many pathogens, the molecules that induce IL-12 production by macrophages and the mechanisms by which they do so remain undefined. Here it is shown that microbial lipoproteins are potent stimulators of IL-12 production by human macrophages, and that induction is mediated by Toll-like receptors (TLRs). Several lipoproteins stimulated TLR-dependent transcription of inducible nitric oxide synthase and the production of nitric oxide, a powerful microbicidal pathway. Activation of TLRs by microbial lipoproteins may initiate innate defense mechanisms against infectious pathogens.

Besides causing disease, mycobacteria have long been recognized for having powerful immunologic adjuvant activity, augmenting both cell-mediated and humoral immune responses. In 1972, a study of the mechanism of mycobacterial adjuvants demonstrated the induction of "soluble mediators," now known to be cytokines, which mediated the augmentation of immune responses (*1*). One cytokine induced by mycobacteria is IL-12 (*2*), a powerful signal for the generation of T helper type 1 lymphocyte (T_H1) responses (*3*) required to eliminate intracellular pathogens (*4*), including *Mycobacterium tuberculosis* (*5*). Furthermore, individuals with mutations in the IL-12 receptor (IL-12R) have increased susceptibility to mycobacterial infection (*6*). We therefore investigated the mycobacterial products that induce IL-12 as well as the mechanism responsible for its induction.

Mycobacterium tuberculosis $H_{37}Rv$ was gamma-irradiated and lysed by mechanical disruption, subcellular fractions were generated (*7*) and tested for the capacity to induce IL-12 with a human monocyte line, THP-1 (*8*). Other than the *M. tuberculosis* lysate, the soluble cell wall–associated proteins (SCWPs) contained most of the IL-12 p40–inducing capacity, consistent with the known adjuvant activity of mycobacterial cell walls (Fig. 1A). The combined cytosolic and membrane protein fraction, the culture filtrate, the lipoglycan from *M. tuberculosis*, and the mycolyl arabinogalactan peptidoglycan complex were less potent on a per weight basis (*9*).

To identify the cell wall–associated proteins responsible for IL-12 p40 release, we fractionated the SCWP preparation by gel filtration chromatography and preparative isoelectric focusing and monitored the bioactivity by measuring IL-12 p40 production (*10*). Subsequent separation of the bioactive fraction by SDS-PAGE indicated four prominently stained proteins: three bands at 17, 21.5, and 39 kD and a doublet at 60 to 70 kD (Fig. 1B). After these regions were transferred to nitrocellulose, they were solubilized (*11*) and used to stimulate THP-1 cells. The band migrating to 21.5 kD induced the highest levels of IL-12 p40 release, followed by the 39-kD band, with the two other regions inducing little activity (Fig. 1C). By using monoclonal antibodies to known *M. tuberculosis* antigens for protein immunoblot analysis, we identified the 21.5-kD band as the 19-kD lipoprotein antigen of *M. tuberculosis*

[1]Department of Microbiology and Immunology, and the [2]Molecular Biology Institute, Divisions of [3]Dermatology and [4]Infectious Disease, Department of Medicine, [5]Howard Hughes Medical Institute, University of California Los Angeles School of Medicine, Los Angeles, CA 90095, USA. [6]Genentech Incorporated, South San Francisco, CA 94080, USA. [7]Department of Microbiology, Colorado State University, Fort Collins, CO 80523, USA. [8]Albert Einstein College of Medicine and Howard Hughes Medical Institute, Bronx, NY 10461, USA. [9]University of Texas Southwestern Medical Center, Dallas, TX 75235, USA. [10]Immunobiology Center, Mount Sinai School of Medicine, New York, NY 10029–6574, USA. [11]Office of the Dean, Harvard School of Public Health, Boston, MA 02115, USA.

*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: rmodlin@mednet.ucla.edu