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*Mycobacterium tuberculosis* Effector Function of IL-22+ CD4+ T Cells

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Membrane-Bound IL-22 after De Novo Production in Tuberculosis and Anti-Mycobacterium tuberculosis Effector Function of IL-22+ CD4+ T Cells

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The role of IL-22–producing CD4+ T cells in intracellular pathogen infections is poorly characterized. IL-22–producing CD4+ T cells may express some effector molecules on the membrane, and therefore synergize or contribute to antimicrobial effector function. This hypothesis cannot be tested by conventional approaches manipulating a single IL-22 cytokine at genetic and protein levels, and IL-22+ T cells cannot be purified for evaluation due to secretion nature of cytokines. In this study, we surprisingly found that upon activation, CD4+ T cells in Mycobacterium tuberculosis-infected macaques or humans could evolve into T effector cells bearing membrane-bound IL-22 after de novo IL-22 production. Membrane-bound IL-22+ CD4+ T effector cells appeared to mature in vivo and sustain membrane distribution in highly inflammatory environments during active M. tuberculosis infection. Near-field scanning optical microscopy/quantum dot-based nanoscale molecular imaging revealed that membrane-bound IL-22, like CD3, distributed in membrane and engaged as ∼100–200 nm nanoclusters or ∼300–600 nm nanodomains for potential interaction with IL-22R. Importantly, purified membrane-bound IL-22+ CD4+ T cells inhibited intracellular M. tuberculosis replication in macrophages. Our findings suggest that IL-22–producing T cells can evolve to retain IL-22 on membrane for prolonged IL-22 t1/2 and to exert efficient cell–cell interaction for anti-M. tuberculosis effector function. The Journal of Immunology, 2011, 187: 000–000.

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aive CD4+ T cells can evolve to at least four distinct cell populations, Th1, Th2, Th17, and induced regulatory T cells, in response to Ag (1, 2). Th17 cell lineage differentiation is regulated by a master transcription factor, retinoid-related orphan receptor γt (1, 2). Murine Th17 cells produce and secrete the signature cytokines IL-17, IL-17F, and IL-22 (1, 2). IL-22 is an IL-10 family cytokine, and is mainly produced by T cells and a subset of NK cells (3–6). Some studies suggest that IL-22 plays a role in development of inflammatory diseases, such as rheumatoid arthritis, psoriasis, and inflammatory bowel diseases (7–11). Other studies implicate that IL-22 may protect against cell injuries (12, 13), and act on IL-22R to facilitate innate immunity against extracellular bacterial and fungi infections (14–17). It is important to note that immune function of Th17 cells or IL-22+ T cells has been investigated through immune manipulation or targeting of IL-17 or IL-22. Given the possibility that lineage differentiation/maturation and in vivo turnover of Th1, Th2, and Th17 cell populations including IL-22+ T effectors are quite plastic and dynamic during infection (1), each of these T effector subsets may represent a heterogeneous cell population producing the IL-17/IL-22 signature cytokine and other effector molecules. From this immunological standpoint, targeting a single IL-17 or IL-22 cytokine in experiments may insufficiently define the function of Th17 T effector cell populations. Use of the IL-17/IL-22 cytokine-expressing Th17 effector cells in experimental investigation may provide additional information regarding the cellular effector function of these cells. However, current biological techniques cannot allow purification or selection of the IL-17/IL-22–producing T effector cell population due to the secretion nature of cytokines, including IL-17/IL-22.

Tuberculosis (TB) remains one of the leading causes for morbidity and mortality worldwide among infectious diseases, with annual 8 million new cases and 2 million deaths (18). Although CD4+ T cells and Th1 cytokines are protective against Mycobacterium tuberculosis infection, full anti-TB immunity appears to involve other undefined immune components (19). Understanding roles of other T cell subsets producing a signature cytokine and other effector molecules should help to elucidate anti-TB immunity and facilitate development of new vaccine and immunotherapeutics against TB. We have recently found that macaque IL-22 transcripts are remarkably increased in lung and lymphoid tissues in severe TB (20), a finding similarly seen in a human study (21). We also demonstrated that whereas most T effector cells secreting conventional cytokines often cannot be directly detected without in vitro Ag restimulation, IL-22 protein produced by macaque T cells could be visualized in situ even in lung tissues containing TB granuloma (22, 23). Moreover, macaque IL-22+ T cells that have matured during M. tuberculosis infection can readily be detected in blood and tissue lymphocytes by intracellular cytokine staining without in vitro Ag restimulation (23). However, immune
function of IL-22–producing CD4^+ T cells in *M. tuberculosis* infection remains unknown.

Our finding that IL-22^+ T effector cells can be readily detected in situ in TB allowed us to hypothesize that IL-22^+ T effector cells or IL-22 play a role in *M. tuberculosis* infection and that IL-22 intracellular trafficking/secrection may differ somehow from most other T cell-producing ILs (24, 25). In testing this hypothesis, we surprisingly found that IL-22 produced by CD4^+ T cells could evolve to retain in cell membrane, and engage as nanoclusters or nanodomains after de novo production in *M. tuberculosis* infection. Notably, membrane-bound IL-22^+ CD4^+ T cells were able to inhibit intracellular *M. tuberculosis* replication in macrophages.

Materials and Methods

**Animals**

Adult rhesus macaques were used in this study. Macaques were naïve prior to *M. tuberculosis* infection on a basis of tuberculin skin test, IFN-γ ELISPOT assays, and thoracic radiographs. *M. tuberculosis*-infected macaques were housed at the Biological Research Resources Annex BSL3 nonhuman primate facilities in University of Illinois and sacrificed after 2 mo of infection. All animal experimental procedures and protocols were approved by the University of Illinois Animal Care Committee.

**M. tuberculosis infection of macaques**

Each monkey was infected with 500 CFU *M. tuberculosis* Erdman (the standard challenge stock from Food and Drug Administration) by the bronchoScope-guided injection of the inoculum into the right caudal lobe, as previously described (23, 26). This was done in the procedure room at the Annex BSL3 nonhuman primate facilities at University of Illinois. The inoculum used for infection was diluted and plated on 7H11 agar plates (BD Pharmingen) to further confirm the bacterial CFU dose for inoculation.

**Isolation of lymphocytes from blood, lung, and spleen**

PBMC were isolated from freshly collected EDTA blood of macaques or humans by Ficoll-Paque Plus (Amer sham, Piscataway, NJ) density gradient centrifugation, as described previously (27). For isolation of lymphocytes from lung and spleen tissues, lung and spleen tissues were minced with sharp scissors and squeezed with sterile copper mesh in Petri dish and collected with RPMI 1640, followed by filtering through 40-μm cell strainer. Mesenteric lymph nodes were carefully teased apart with 18-gauge needle in Petri dish with RPMI 1640 to form single-cell suspension. Collected cell suspensions from lung, spleen, and mesenteric lymph nodes were further purified by Ficoll-Paque Plus density gradient centrifugation to collect pure lymphocytes. The freshly isolated lymphocytes from blood, lung, spleen, and mesenteric lymph nodes were stained by trypan blue to examine the viability and number cell counts. The lymphocytes were finally suspended into 10% FCS–RPMI 1640 media with a concentration of 10^6 cells/ml for further study.

**Flow cytometry analysis of macaque or human CD4 T cell precursors producing membrane-bound IL-22 and of IL-22R-expressing macrophages**

PBMC that isolated freshly from the blood of *M. tuberculosis*-infected macaques or purified protein derivative (PPD)^+ or PPD^− human donors were incubated with *M. tuberculosis* in 96-well plates in multiplicity of infection (MOI) = 1 for 18 h. Then cells were washed with 2% FBS/PBS and stained with anti-human IL-22 Ab pretreatment of IL-22^+ T cells could block the immunofluorescence staining of membrane-bound IL-22^+ T cells by fluorescence IL-22–conjugated Ab (see above). In addition, matched normal serum or isotype IgG served as negative controls for staining cytokines or surface markers. We did not see nonspecific immune staining of IL-22 as evaluated by flow cytometry, confocal imaging, or near-field scanning optical microscopy (NSOM/quantum dot (QD)-based molecular imaging (see below)). Furthermore, to ensure the specific immune staining of membrane-bound IL-22 in *M. tuberculosis* infection, PBMC were obtained biweekly for 8 wk from three healthy uninfected and five simian HIV–infected cynomolgus macaques, and assessed for IL-22–producing T cells using the immune staining assay, as described above. No or very few membrane-bound IL-22^+ CD4^+ T cells (∼10−3 peptide peptides spanning Ag85B and ESAT6 (GenScript), anti-CD28 (CD28.2; BD Pharmingen), and anti-CD49d (9F10; BD Pharmingen) for 1 h. After thoroughly washing, these cells were used for Ab staining without treatment with membrane permeabilization reagents. Cells were incubated with FITC CD4 (L200; BD Pharmingen), PE/Cy7 CD3 (SP34-2; BD Pharmingen), and IL-22–biotinylated or PB (goat anti-human; R&D Systems) Abs for 30 min. After thoroughly washing, cells were incubated with streptavidin PB (Invitrogen). These cells were then washed twice with 2% FBS/PBS, fixed with 2% formalin/PBS, and then analyzed by flow cytometry. To investigate whether secretory form of IL-22 also existed, IL-22 in culture supernatants from activated, enriched CD4^+ lymphocytes or IL-22^+ CD4^+ T cells was examined using the dot blot assay, as we described (28, 29). We used the dot blot assay, as ELISA assay for quantitation of macaque IL-22 was not commercially available. Typically, ∼1 × 10^6 enriched CD4^+ T cells from spleens of infected macaques were stimulated by PPD in culture for 2–4 d. Supernatants were collected, either unconcentrated or concentrated 50× using Vivacell concentrator (Vivasciences), as we described (28, 29), and then tested for IL-22 by the dot blot assay (28, 29) using the same anti–IL-22 Ab, as described above. Negative control was culture supernatants from unstimulated PBL of uninfected macaques. The specificity of IL-22 Ab staining was confirmed repeatedly using matched normal serum, control Abs, or control animals. No or very few IL-22–producing CD4^+ T cells were detectable in the longitudinal control experiments (data not shown).

Flow cytometry analysis of membrane-bound IL-22 produced by lymphocytes that isolated from the spleens or lungs of *M. tuberculosis*-infected macaques without additional in vitro Ag restimulation

Cells that isolated freshly from the spleens or lungs of monkeys after 2-mo infection were stained directly with Abs without additional in vitro treatment of Ag or Abs or membrane permeabilization reagents. Cells were then incubated with FITC CD4 (L200; BD Pharmingen), PE/Cy7 CD3 (SP34-2; BD Pharmingen), and IL-22–biotinylated (goat anti-human; R&D Systems) Abs for 30 min. After thoroughly washing, cells were incubated with streptavidin PB (Invitrogen). These cells then were washed twice with 2% FBS/PBS, fixed with 2% formalin/PBS, and analyzed by flow cytometry.

**Experimental controls for assessing membrane-bound IL-22^+ CD4^+ T cells**

We found that macaque and human IL-2 shared >98% similarity in amino acid sequence (data not shown), and that the anti-human IL-2 Ab could readily cross-react with soluble macaque IL-22 produced either by *Lactococcus lactis* (30) or *Listeria monocytogenes* expression system on dot blot or Western blot assay (data not shown). Consistently, nonconjugated anti-human IL-22 Ab pretreatment of IL-22^+ T cells could block the immune staining of membrane-bound IL-22^+ T cells by fluorescence IL-22–biotinylated Ab (see above). In addition, matched normal serum or isotype IgG served as negative controls for staining cytokines or surface markers.

**NSOM/QD-based nanoscale analysis of membrane-bound IL-22 produced by spleen lymphocytes that isolated from *M. tuberculosis*-infected macaques**

Cells that isolated from the spleens of monkeys after 8-wk infection of *M. tuberculosis* were fixed with 2% formalin/PBS first. Our previous works have shown that short-term 2% formalin/PBS would greatly reduce unspecific activation during Ab staining, but would not induce significant artificial cellular nanostructures (31–33). These cells then were washed extensively with 2% FBS/PBS and ready for Ab staining. Cells were incubated with mouse anti-human CD4 (L200; BD Pharmingen) and goat anti-human CD28 Ab (R&D Systems) Ab for 30 min. After washing with 2% FBS/PBS, these cells were stained with streptavidin-QD655 (Invitrogen) and QD605 goat anti-mouse IgG (Invitrogen). After incubating with all the Abs or fluorochromes, cells were fixed with 2%...
formalin/PBS and washed thoroughly again using 2% FBS/PBS. Cells then were washed again with dd water twice to remove the salt in the PBS. NSOM (Veeco, Aurora 3.0) imaging was done, as described in our previous work (30).

Confocal microscopic analysis of membrane-bound IL-22 produced by spleen lymphocytes of M. tuberculosis-infected macaques

Cells that isolated from the spleens of monkeys after 8-wk infection of M. tuberculosis were washed extensively with 2% FBS/PBS and incubated with mouse anti-human FITC CD4 (L200; BD Pharmingen) and goat anti-human IL-22–biotinylated (R&D Systems) Ab for 30 min. After washing with 2% FBS/PBS, these cells were stained with streptavidin-QD655 (Invitrogen). After incubating with all the Abs or fluorochromes, cells were fixed with 2% formalin/PBS and washed thoroughly again using 2% FBS/PBS. Cells then were washed again with dd water twice to remove the salt in the PBS. Confocal microscopic imaging was done, as described in our previous work (30).

In situ confocal microscopic analysis of IL-22 in lung tissue sections of M. tuberculosis-infected macaques

The ≈5-μm-thick frozen lung sections were prepared, as we recently described (34), from optimal cutt ing temperature compound (OCT)-embedded lung tissues from healthy macaques or M. tuberculosis-infected macaques. Tissue sections were first incubated overnight in a wet box with polyclonal rabbit anti-human IL-22 (N terminus; Capralogues) and monoclonal mouse anti-human CD3 (F7.2.38; DakoCytomation) or isotype control IgG or normal rabbit serum. After thoroughly washed with PBS, tissue sections were fixed with 2% formalin/PBS and washed thoroughly with PBS. Tissue sections were then incubated with FITC-conjugated donkey anti-rabbit IgG (BioLegend) and Cy3-conjugated goat anti-mouse IgG (BioLegend), followed by thorough PBS washing. Next, tissue sections were fixed gently with 2% formalin/PBS again and washed thoroughly with dd water to remove the salt in PBS. Finally, tissue sections were mounted on slides using fluorescence mounting medium with DAPI for confocal microscope (Zeiss; LSM 510, 63 NA) imaging.

In situ NSOM/QD-based nanoscale analysis of IL-22 in lung tissue sections of M. tuberculosis-infected macaques

The tissue preparation and the primary Ab staining are the same for the confocal microscopic imaging. After primary Ab staining, tissue sections were washed and incubated with QD655-conjugated goat anti-mouse secondary Ab and QD605-conjugated goat anti-rabbit secondary Ab. These tissue sections were then washed thoroughly, fixed with 2% formalin/ PBS again, and washed thoroughly with dd water to remove the salt in PBS. Then these tissue sections were imaged with dual-color NSOM/QD-based nanoscale imaging system (Veeco, Aurora 3.0).

Isolation of monocytes and membrane-bound IL-22+ CD4+ T cells

PBMC were isolated from the blood of M. tuberculosis-infected macaques, and monocytes were obtained by adherence purification on plastic plates (Falcon), as described previously (27). The plates were washed after 2 h of adherence, and monocytes were detached by cold 2% FBS/PBS. Nonadherent cell fraction containing T and B cells were stimulated with PPD in the presence of anti-CD3/anti-CD28 Abs (BD Pharmingen) to generate large numbers of membrane-bound IL-22+ CD4+ T cells. After overnight culture, the isolated T cells were stained with anti-IL-22 PE (R&D Systems), followed by anti-PE magnetic beads (Miltenyi Biotec). The stained live cells were then loaded to the purification column following instructions from the manufacturer. The passing fraction was collected as IL-22- (negative) cells that did not bear membrane-bound IL-22; IL-22+ T cells held by anti-PE magnetic beads were then released by releasing buffer (Miltenyi Biotec). The isolated IL-22+ T cells were stained again with anti-CD4 FITC (BD Pharmingen), followed by anti-FITC magnetic microbeads for secondary purification.

In vitro M. tuberculosis infection of monocyte-derived macrophages or immature DCs and intracellular M. tuberculosis growth assay

Autologous monocytes (5 × 10^6/well) were cultured in round-bottom 96-well plates with 10% FBS–RPMI 1640 medium in the presence of human rIL-4 (BD Pharmingen) and GM-CSF (Sigma-Aldrich) for 4 d, as previously described (35). Supernatants were then removed, and M. tuberculosis inoculum was added at a MOI = 1. After 3 h of incubation at 37°C, supernatants were aspirated and each well was washed three times to remove noningested mycobacteria. Enriched IL-22+ CD4+ T cells (5 × 10^5) were incubated with anti-IL-22 neutralizing Ab (10 μg/ml, 142928; R&D Systems) or mouse IgG (10 μg/ml) and cultured in the presence of each of them, as indicated in Results. Autologous, bead-enriched CD20+ B cells, CD25+ CD4+ T cells, or IL-22+ CD4+ T cells (5 × 10^5 for each subset) served as a control. For the noncontact setting in Transwell culture system (Corning) experiments, enriched IL-22+ CD4+ T cells (5 × 10^5) were placed in the upper chamber of Transwell culture system without physical contact with M. tuberculosis-infected macrophages (5 × 10^5) that were cultured in the lower chamber. Therefore, noncontact IL-22+ CD4+ T cells were not able to contact macrophages, but secretory IL-22 could trans-pass through the microholes and reach macrophages in the lower chamber. Secretory IL-22 was detectable in the concentrated culture me- dium. For the contact setting, enriched IL-22+ CD4+ T cells and infected macrophages were cocultured in the lower chamber of Transwell culture system. After culturing in 5% CO2 at 37°C for 4 d, wells were aspirated, and 100 μl lysis buffer (0.067% SDS in Middlebrook 7H9) was added to each well. Plates were incubated at 37°C for 10 min, followed by neutralization of SDS with 300 μl PBS with 20% BSA. Lysates from each well were pooled, and two 10-fold serial dilutions of lysate in 7H9 medium were made. Aliquots of each dilution of lysate and supernatant were plated onto Middlebrook 7H10 agar and incubated for 3 wk in 5% CO2 at 37°C until colonies were large enough to be counted. GM-CSF/Human IL-4 treatment alone only induced background level of IL-22R expression, but subsequent M. tuberculosis infection of macrophages or immature DC induced apparent IL-22R expression (data not shown).

Statistical analysis

Statistical analysis was done using Student t test, as previously described (30). A p value <0.05 was considered statistically significant.

Results

Upon activation, CD4+ T cells in M. tuberculosis-infected macaques and humans differentiated into T effector cells bearing membrane-bound IL-22 after de novo IL-22 production

Although IL-22, like most other cytokines, should be secretory (21, 36), cell membrane-bound IL-22 after de novo production may enjoy longer t1/2 and exert better effector effects on IL-22R+ target cells via surface–molecule interactions between a IL-22+ T cell and a IL-22R+ cell. The notion for membrane-bound IL-22 appears consistent with our recent observation indicating that IL-22+ T cells matured during M. tuberculosis infection could directly be detected in situ even in lung tissues or directly by intracellular IL-22 staining (22, 23). We therefore hypothesized that TB-induced IL-22+ T cells could have their IL-22 to retain in cell membrane after de novo production, and thus readily be detected by surface immune staining. To test this hypothesis, PBMC from M. tuberculosis-infected macaques were cultured with M. tuberculosis for activation, and assessed for cell membrane-bound IL-22+ T effector cells after de novo IL-22 production using cell surface cytokine staining that skipped the treatment with cell membrane permeabilization reagents. Interestingly, significant numbers of CD4+ T effector cells bearing membrane-bound IL-22 were detected in PBMC from M. tuberculosis-infected macaques, but not from uninfected ones (Fig. 1A, 1C). In contrast, very few membrane-bound IFN-γ+ T cells were detectable by direct cell surface staining in PBMC from the same macaques (Fig. 1A, 1C). Consistently, appreciable numbers of CD4+ T cells bearing membrane-bound IL-22, but not IFN-γ, were also detected by direct cell surface staining after M. tuberculosis stimulation of PBMC from latently M. tuberculosis-infected humans (Fig. 1B, 1D). We did not observe any coexpression of Th1 cytokines by IL-22+ CD4+ T cells (Fig. 1E). The membrane-bound IL-22 could also be seen by fluorescence membrane imaging (Fig. 1F), although secretory form of IL-22 also existed in culture supernatants of activated CD4+ T effector cells (Fig. 1G). These results therefore suggested that significant num-
MOI = 1 for 18 h prior to analysis. M. tuberculosis membrane-bound IFN-γ upon activation by CD3/CD28 Ab (data not shown). A representative flow cytometry histograms show that IL-22–producing macrophages could also express membrane-bound IL-22 upon activation by CD3/CD28 Ab (data not shown). A, Representative flow cytometry histograms show that M. tuberculosis stimulation of PBMC from M. tuberculosis-infected macrophages induced membrane-bound IL-22. CD4+ T effector cells, whereas CD4+ T cells in PBMC from uninfected macrophages contained very few membrane-bound IL-22+ T effectors. PBMC from infected macrophages were isolated from the blood collected 2 mo after M. tuberculosis infection. Only 0.18% of CD4+ T cells expressing membrane-bound IFN-γ was detectable in M. tuberculosis-infected PBMC. PBMC of macrophages was infected with M. tuberculosis in 96-well plates in MOI = 1 for 18 h prior to analysis. B, Representative flow cytometry histograms show that M. tuberculosis stimulation of PBMC from PPD+ human donors induced 12% of CD4+ T cells bearing membrane-bound IL-22. Only ~0.3% of CD4+ T cells bearing membrane-bound IL-22 were detected in PBMC isolated from PPD- donors. Also, only ~0.01% of CD4+ T cells expressing membrane-bound IFN-γ could be detected in blood lymphocytes of PPD+ donors. Human PBMC were similarly infected with M. tuberculosis, as described above, or stimulated overnight with anti-CD3/anti-CD28 in presence of PPD and stained without cell permeabilization treatment. C, Bar graph data show that M. tuberculosis-infected macrophages exhibited significantly greater numbers of CD4+ T cells bearing membrane-bound IL-22 than uninfected macrophages after M. tuberculosis stimulation in vitro (***p < 0.001, n = 3). Data are mean percentage numbers among CD4+ T cells with error bars of SEM derived from three different donors. D, Bar graph data show that PPD+ human donors exhibited significantly greater numbers of CD4+ T cells bearing membrane-bound IL-22 than PPD- donors after M. tuberculosis stimulation in vitro (**p < 0.01, n = 3). Data are mean percentage numbers among CD4+ T cells with error bars of SEM derived from three different donors. E, A representative flow cytometry data showing that IL-22–producing CD4+ T cells do not coexpress TGF-α (CD4-gated). No coexpression of IFN-γ was found either (data not shown). Cells were isolated from peripheral blood of M. tuberculosis-infected macrophages. Similar data were observed in five different macrophages. F, A representative confocal microscopic image shows that IL-22 forms the capping domain (as marked by white arrowheads) and colocalizes in part with CD4 on T cell membrane. G, IL-22 in concentrated culture supernatant (left) from activated, enriched CD4+ T cells could be detected by the dot blot assay. CD4+ T cells from spleens of M. tuberculosis-infected macrophages were enriched by deleting CD8+ cells using immunomagnetic beads, as described above. Approximately 1 × 10^6 enriched CD4+ T cells were stimulated by PPD in culture for 2 d. Supernatants were collected, either unconcentrated or concentrated 50× using Vivacell concentrator (Vivasciences) (28, 29), and then tested for IL-22 by the dot plot assay, as we described (28, 29). Negative control (right) was culture supernatant from unstimulated PBL of uninfected macrophages.

Membrane-bound IL-22+ CD4+ T cells matured in vivo and sustained in highly inflammatory environments during active M. tuberculosis infection

We then asked a question as to whether membrane-bound IL-22+ T effector cells could mature and sustain in vivo under highly inflammatory environments during active M. tuberculosis infection. To address this straightforward question, macaques were infected with M. tuberculosis by bronchoscope-guided inoculation, as previously described (35), and assessed directly for membrane-bound IL-22+ CD4+ T cells using surface immune staining without in vitro stimulation and membrane permeabilization. Surprisingly, appreciable percentage numbers (0.82 ± 0.09%) of spleen CD4+ T cells bearing membrane-bound IL-22 could be detected without in vitro culture and membrane permeabilization treatment (Fig. 2). A short-term in vitro restimulation of spleen lymphocytes by pooled ESAT-6 and Ag85B peptides resulted in the increased percentage numbers (3.03 ± 0.66%) of membrane-bound IL-22+ CD4+ T cells, suggesting that IL-22 binds to and retains in membrane after de novo production in newly generated IL-22+ T effector cells (Fig. 2). Consistently, the mature CD4+ T effector cells bearing membrane-bound IL-22 appeared to be driven by bacterial Ag loads in vivo because greater percentages (3.91 ± 1.66%) of these effector T cells could be detected directly without Ag restimulation in lymphocytes isolated from M. tuberculosis-infected lung tissues of the same macaques in comparisons with blood and spleen lymphocytes (Fig. 2) (37). The number of membrane-bound IL-22+ T cells from the lung was only subtly increased after the in vitro Ag stimulation (4.67 ± 1.88%) (Fig. 2), suggesting that most of these lung IL-22+ T cells matured enough in vivo and sustained their capability to retain IL-22 on membrane without rapid degradation after de

Beginning of FIGURE 1. Upon activation, CD4+ T cells in M. tuberculosis-infected macaques and humans differentiated into T effector cells bearing membrane-bound IL-22 after de novo IL-22 production. Percentage numbers of CD4+ T cells bearing membrane-bound IL-22 or CD4+ T cells expressing membrane-bound IFN-γ are shown in the upper right quadruple of flow cytometry histograms. Data were gated on CD4 T cells. All cells were surface stained directly without treatment with membrane permeabilization reagents. No membrane-bound IL-22 were detected for negative controls (see Materials and Methods). Membrane-bound IL-22 did not appear to be specific for M. tuberculosis infection as CD4+ T cells from Listeria-vaccinated macaques could also express membrane-bound IL-22 upon activation by CD3/CD28 Ab (data not shown). A, Representative flow cytometry histograms show that M. tuberculosis stimulation of PBMC from M. tuberculosis-infected macrophages induced membrane-bound IL-22. CD4+ T effector cells, whereas CD4+ T cells in PBMC from uninfected macaques contained very few membrane-bound IL-22+ T effectors. PBMC from infected macrophages were isolated from the blood collected 2 mo after M. tuberculosis infection. Only 0.18% of CD4+ T cells expressing membrane-bound IFN-γ was detectable in M. tuberculosis-infected PBMC. PBMC of macrophages were infected with M. tuberculosis in 96-well plates in MOI = 1 for 18 h prior to analysis. B, Representative flow cytometry histograms show that M. tuberculosis stimulation of PBMC from PPD+ human donors induced 12% of CD4+ T cells bearing membrane-bound IL-22. Only ~0.3% of CD4+ T cells bearing membrane-bound IL-22 were detected in PBMC isolated from PPD- donors. Also, only ~0.01% of CD4+ T cells expressing membrane-bound IFN-γ could be detected in blood lymphocytes of PPD+ donors. Human PBMC were similarly infected with M. tuberculosis, as described above, or stimulated overnight with anti-CD3/anti-CD28 in presence of PPD and stained without cell permeabilization treatment. C, Bar graph data show that M. tuberculosis-infected macrophages exhibited significantly greater numbers of CD4+ T cells bearing membrane-bound IL-22 than uninfected macrophages after M. tuberculosis stimulation in vitro (***p < 0.001, n = 3). Data are mean percentage numbers among CD4+ T cells with error bars of SEM derived from three different donors. D, Bar graph data show that PPD+ human donors exhibited significantly greater numbers of CD4+ T cells bearing membrane-bound IL-22 than PPD- donors after M. tuberculosis stimulation in vitro (**p < 0.01, n = 3). Data are mean percentage numbers among CD4+ T cells with error bars of SEM derived from three different donors. E, A representative flow cytometry data showing that IL-22–producing CD4+ T cells do not coexpress TGF-α (CD4-gated). No coexpression of IFN-γ was found either (data not shown). Cells were isolated from peripheral blood of M. tuberculosis-infected macrophages. Similar data were observed in five different macrophages. F, A representative confocal microscopic image shows that IL-22 forms the capping domain (as marked by white arrowheads) and colocalizes in part with CD4 on T cell membrane. G, IL-22 in concentrated culture supernatant (left) from activated, enriched CD4+ T cells could be detected by the dot blot assay. CD4+ T cells from spleens of M. tuberculosis-infected macrophages were enriched by deleting CD8+ cells using immunomagnetic beads, as described above. Approximately 1 x 10^6 enriched CD4+ T cells were stimulated by PPD in culture for 2 d. Supernatants were collected, either unconcentrated or concentrated 50× using Vivacell concentrator (Vivasciences) (28, 29), and then tested for IL-22 by the dot plot assay, as we described (28, 29). Negative control (right) was culture supernatant from unstimulated PBL of uninfected macrophages.
Membrane-bound IL-22 molecules distributed in membrane and engaged as ~100–200 nm nanoclusters or ~300–600 nm high-density nanodomains on membrane of IL-22+ CD4+ T effectors

Studies from us and others suggest that during cellular signaling/activation, cell membrane ligands or receptors undergo instructed engagements forming clusters or capping domains to enhance ligand–receptor interaction between the two contact cells and to sustain cellular activation (32, 38, 39). Thus, we hypothesized that membrane-bound IL-22 molecules might engage themselves, forming unique nanostructures for putative interaction with IL-22R on responsive cells. To test this hypothesis, we employed our expertise of confocal imaging and NSOM/QD-based nanoscale molecular imaging (30, 32, 39) to visualize membrane-bound nature and nanostructures of IL-22 molecules. Confocal microscopy showed that IL-22 on the surface of spleen CD4+ T effectors localized as large capping domains (Fig. 3A), but could not confer high-resolution nanoscale imaging of molecular details. In contrast, our novel NSOM/QD-based nanoscale imaging system, which exclusively images cell surface membrane molecules, but not intracellular contents of intact cells (30, 32, 39), revealed that IL-22 cytokine proteins engaged themselves as ~100–200 nm nanoclusters on outer membrane (Fig. 3B). Some of these IL-22 nanoclusters were arrayed to form ~300–600 nm nanodomains (Fig. 3B, 3C). The formation of these IL-22 nanoclusters or nanodomains on membrane suggested that large amounts of IL-22 were retained on T cell membrane and actively involved in immune responses during active M. tuberculosis infection. However, these membrane-bound IL-22 nanoclusters were seldom colocalized with CD4 molecules (Fig. 3B, merge).

To examine whether IL-22 was distributed within membrane rather than simply attached to the very surface of membrane, we dissected cross-sectional membrane and intracellular compartments of individual IL-22+ T effector cells in lung tissues containing TB granulomas using in situ confocal microscopy and NSOM/QD imaging techniques. Confocal images showed that IL-22, but not IFN-γ or IL-4, could be reproducibly detected on CD3+ T cells in the sections derived from lung tissues containing TB granulomas (Fig. 4A). Under confocal microscopy, IL-22 and CD3 appeared to exhibit similar membrane distribution in the lung sections (Fig. 4A). Consistently, NSOM/QD-based nanoscale imaging showed that IL-22 cytokine proteins were distributed as nanoclusters/nanodomains in membrane rather than in the cytoplasm of effector cells in the lung sections (Fig. 4B–D). IL-22 indeed shared distribution patterns with membrane CD3 molecules (Fig. 4B). Some IL-22 nanoclusters appeared to colocalize with CD3 nanoclusters (Fig. 4B). Collectively, these results suggest that membrane-bound IL-22 molecules distributed in membrane and engaged as ~100–200 nm nanoclusters or ~300–600 nm high-density nanodomains on membrane of IL-22+ CD4+ T effectors in M. tuberculosis infection.

Membrane-bound IL-22+ CD4+ T cells inhibited intracellular M. tuberculosis replication in macrophages

The role of IL-22–producing CD4 T effector cells in M. tuberculosis infection remains unknown. Now, our identification of CD4+ T effector cells bearing membrane-bound IL-22 made it possible to isolate IL-22+ CD4+ T cells and to determine whether these cells could have positive or negative impact in intracellular M. tuberculosis replication in IL-22R–expressing macrophages or DC (Fig. 5A). Thus, monocye-derived macrophages or DCs were infected with M. tuberculosis, and cocultured with autologous membrane-bound IL-22+ CD4+ T cells that were purified from macroage PBL using immunomagnetic beads, as described previously (35, 39). This procedure enriched membrane-bound IL-22+ CD4+ T cells to the level of 70–96% (Fig. 5B). Purified autologous B cells, CD25+ CD4+ T cells, and IL-22+ CD4+ T cells served as controls, respectively. Interestingly, the purified membrane-bound IL-22+ CD4+ T cells significantly inhibited intracellular M. tuberculosis replication in cultured macrophages (Fig. 5C). Such inhibition of M. tuberculosis replication appeared to be mediated at least in part by membrane-bound IL-22 as anti–IL-22 neutralizing Ab could significantly reverse the inhibition (Fig. 5C) and as the intracellular M. tuberculosis growth was inhibited only by membrane IL-22+ CD4+ T cells contacting with...
Membrane-bound IL-22 molecules were engaged as $\sim 100–200$ nm nanoclusters or $\sim 300–600$ nm high-density nanodomains on membrane of IL-22$^+$ CD4$^+$ T effectors. No membrane-bound IL-22 were detected for negative controls (see Materials and Methods).

A, Confocal microscopic images show that membrane-bound IL-22 formed capping domains on CD4 T cells after in vivo M. tuberculosis infection. Lymphocytes isolated from the spleens of M. tuberculosis-infected macaques (2 mo postinfection) were surface stained with anti–IL-22 Ab, without in vitro Ag stimulation or membrane permeabilization. This only allowed membrane-bound IL-22 to be stained and visualized by confocal microscopy. The white arrowheads indicated membrane-bound IL-22 on CD4$^+$ T cells. Note that membrane-bound IL-22 forms capping domains on CD4$^+$ T cell surface. No membrane-bound IL-22 was observed in PBMC from uninfected macaques (data not shown). Isotype control IgG did not stain cell membrane of PBMC from infected macaques (data not shown).

Scale bars, 5 µm. B, Representative NSOM/QD-based nanoscale imaging shows that membrane-bound IL-22 are engaged as $\sim 100–200$ nm nanoclusters or as $\sim 300–600$ nm high-density nanodomains. Upper panels, Show fluorescence imaging of two-color molecular staining of IL-22 (green) and CD4 (red) on outer membrane and representative topography (right) for one representative membrane-bound IL-22$^+$ CD4$^+$ T effector cell. Note that NSOM/QD-based nanoscale imaging only detects fluorescence dots on outer membrane, but not underneath (see text). Few IL-22 molecules colocalize with CD4 in the merged image. Lower panels, Show the enlarged images derived from rescanning the dashed squares in the IL-22 and CD4 images and topography in the upper panels. The scale bars for the upper and lower panels are 3.214 µm and 385 nm, respectively. The staining samples for NSOM/QD imaging were similarly prepared as those for confocal imaging. Integration time for all the images is 30 ms with 400 x 400 scanning lines. C, Upper panel, A representative fluorescent intensity profile of an IL-22 nanocluster. The fluorescent intensity profile (upper) of the cross section of the objects is marked by a line in the fluorescence image. Shown is an IL-22 nanocluster with diameters of 274 nm (full width at half maximum [FWHM]). Lower panel, Histogram graph shows the frequency of different sizes of FWHM of IL-22 nanoclusters on the CD4$^+$ T cells. The green arrowheads marked the FWHM of IL-22 microdomains ($>500$ nm). D, Upper panel, A representative fluorescence intensity profile of a CD4 nanocluster. The fluorescent intensity profile (upper) of the cross section of the objects is marked by a line in the fluorescence image as well. Shown are CD4 clusters with diameters of 87 nm (FWHM). Lower panel, Histogram graph shows the frequency of different sizes of FWHM of CD4 nanoclusters or nanodomains on the CD4$^+$ T cells.
M. tuberculosis-infected macrophages, but not by those non-contacting IL-22+ CD4+ T cells or culture medium containing IL-22 secreted by the effector cells (Fig. 5D). The in vitro work therefore provided novel evidence demonstrating that membrane-bound IL-22+ CD4+ T cells could inhibit intracellular M. tuberculosis replication in macrophages.

Discussion

In the current study, we made an unexpected discovery that IL-22-producing CD4+ T cells in M. tuberculosis-infected macaques and humans could retain or hold IL-22 in the cell membrane after de novo production. This observation was confirmed by flow cytometry-based surface immune staining, confocal microscopy, and, most importantly, NSOM/QD-based nanoscale imaging. The NSOM/QD-based nanoscale imaging on cell surface indeed provides most direct evidence for the membrane-bound IL-22 as NSOM scans and detects molecules or signals exclusively on the cell surface, but not the underneath when cell surface is scanned (32, 39).

NSOM/QD imaging of cross-sectional membrane in tissue sections also suggests that membrane IL-22 molecules are distributed in membrane sharing distribution patterns with membrane CD3 molecules of T cells. IL-22 is therefore one of the very few cytokines with both secretory and membrane-bound forms (24, 25), although conventional protein chemistry and immunology studies suggest that the mature IL-22 protein, like other cytokines, is most likely secreted to the extracellular environment (40). Nevertheless, low-level secretory human or macaque IL-22 proteins appeared to exist as well in TB bronchoalveolar lavage fluid and culture medium after in vitro immune stimulation (21) (Fig. 1G). The precise mechanism whereby IL-22 retains in the membrane is currently not known. Both human and macaque mature IL-22 do not carry classical trans-membrane sequences, and we found no evidence that TB could induce variant IL-22 mRNA encoding additional trans-membrane domains in the C terminal (data not shown). It is important to note that membrane-bound IL-22+ CD4+ T cells can directly be detected without in vitro Ag stimulation in spleen cells.
CD14+ macrophages (gated on CD14) were observed in monocyte-derived macrophages without derived macrophages was inhibited by contact IL-22 + CD4+ T cells (contacting with infected macrophages), but not those noncontact IL-22 + CD4+ T cells. IL-22R and CD14, and analyzed by flow cytometry. Left panel, A representative flow cytometry histogram shows that only 1.5% of IL-22R-expressing CD14+ macrophages (gated on CD14) were observed in monocyte-derived macrophages without M. tuberculosis infection. Right panel, A representative flow cytometry histogram shows that after in vitro M. tuberculosis infection, ~18% of CD14+ macrophages (gated on CD14) expressed IL-22R (upper right quadruple). Similar results were seen in other five independent experiments. B, Representative flow cytometry data show that immunomagnetic bead-based purification could enrich IL-22+ CD4+ T cells to the level of 70–96%. Similar results were observed in six other repeated experiments. C, Bar graphic data show that IL-22+ CD4+ T cells inhibited intracellular M. tuberculosis replication in monocyte-derived macrophages. M. tuberculosis-infected macrophages (5 × 10⁵) were cocultured with medium alone, CD20+ B cells (5 × 10⁵), CD25+ CD4+ T cells (5 × 10⁵), IL-22+ CD4+ T cells (5 × 10⁵), or IL-22- CD4+ T cells (5 × 10⁵) plus anti–IL-22 Ab (10 μg/ml), IL-22+ CD4+ T cells (5 × 10⁵) plus IgG (10 μg/ml) for 4 d. The cultured cells were lysed, and CFU counts in lysate were determined, as described in Materials and Methods. Enriched IL-22+ CD4+ T cells (5 × 10⁵) significantly reduced M. tuberculosis CFU counts when compared with control cultures (**p < 0.001, ***p < 0.01, *p < 0.05, respectively). Blocking of membrane-bound IL-22 by anti–IL-22 Ab, but not isotype IgG control from the beginning throughout the assay led to a reduced capability of IL-22+ CD4+ T cells to inhibit intracellular M. tuberculosis (*p < 0.05). Data were mean values with error bars of SEM derived from four rhesus macaques in five independent experiments. The marked reduction in CFU counts was interpreted as “inhibition” in the text, although “killing” was implicated based on the growth kinetics of M. tuberculosis on days 0, 2, and 4 postinfection (data not shown). D, Bar graphic data show that M. tuberculosis growth in monocyte-derived macrophages was inhibited by contact IL-22+ CD4+ T cells (contacting with infected macrophages), but not those noncontact IL-22+ CD4+ T cells or culture medium containing IL-22 secreted by the IL-22+ CD4+ T effector cells. M. tuberculosis-infected macrophages (5 × 10⁵) were cocultured in Transwell system with medium alone, contact IL-22+ CD4+ T cells (5 × 10⁵), and noncontact IL-22+ CD4+ T cells (5 × 10⁵) for 4 d prior to bacteria counts. Noncontact means that enriched IL-22+ CD4+ T cells were placed in the upper chamber of Transwell culture system without physical contact with infected macrophages that were cultured in the lower chamber. Therefore, noncontact IL-22+ CD4+ T cells did not contact macrophages, but secretory IL-22 in the medium could trans-pass through the microholes and reach macrophages in the lower chamber. Contact means that IL-22+ CD4+ T cells and infected macrophages were cocultured in the lower chamber of Transwell culture system. Data in graph were mean values with error bars of SEM derived from four rhesus macaques. **p < 0.01. NS, no significant difference.

FIGURE 5. Membrane-bound IL-22+ CD4+ T cells inhibited intracellular M. tuberculosis replication in monocyte-derived macrophages. A, Flow cytometry characterization of IL-22R expression induced by M. tuberculosis infection of macrophages. Monocyte-derived macrophages were stained with IL-22R and CD14, and analyzed by flow cytometry. Left panel, A representative flow cytometry histogram shows that only 1.5% of IL-22R-expressing CD14+ macrophages (gated on CD14) were observed in monocyte-derived macrophages without M. tuberculosis infection. Right panel, A representative flow cytometry histogram shows that after in vitro M. tuberculosis infection, ~18% of CD14+ macrophages (gated on CD14) expressed IL-22R (upper right quadruple). Similar results were seen in other five independent experiments. B, Representative flow cytometry data show that immunomagnetic bead-based purification could enrich IL-22+ CD4+ T cells to the level of 70–96%. Similar results were observed in six other repeated experiments. C, Bar graphic data show that IL-22+ CD4+ T cells inhibited intracellular M. tuberculosis replication in monocyte-derived macrophages. M. tuberculosis-infected macrophages (5 × 10⁵) were cocultured with medium alone, CD20+ B cells (5 × 10⁵), CD25+ CD4+ T cells (5 × 10⁵), IL-22+ CD4+ T cells (5 × 10⁵), or IL-22- CD4+ T cells (5 × 10⁵) plus anti–IL-22 Ab (10 μg/ml), IL-22+ CD4+ T cells (5 × 10⁵) plus IgG (10 μg/ml) for 4 d. The cultured cells were lysed, and CFU counts in lysate were determined, as described in Materials and Methods. Enriched IL-22+ CD4+ T cells (5 × 10⁵) significantly reduced M. tuberculosis CFU counts when compared with control cultures (**p < 0.001, ***p < 0.01, *p < 0.05, respectively). Blocking of membrane-bound IL-22 by anti–IL-22 Ab, but not isotype IgG control from the beginning throughout the assay led to a reduced capability of IL-22+ CD4+ T cells to inhibit intracellular M. tuberculosis (*p < 0.05). Data were mean values with error bars of SEM derived from four rhesus macaques in five independent experiments. The marked reduction in CFU counts was interpreted as “inhibition” in the text, although “killing” was implicated based on the growth kinetics of M. tuberculosis on days 0, 2, and 4 postinfection (data not shown). D, Bar graphic data show that M. tuberculosis growth in monocyte-derived macrophages was inhibited by contact IL-22+ CD4+ T cells (contacting with infected macrophages), but not those noncontact IL-22+ CD4+ T cells or culture medium containing IL-22 secreted by the IL-22+ CD4+ T effector cells. M. tuberculosis-infected macrophages (5 × 10⁵) were cocultured in Transwell system with medium alone, contact IL-22+ CD4+ T cells (5 × 10⁵), and noncontact IL-22+ CD4+ T cells (5 × 10⁵) for 4 d prior to bacteria counts. Noncontact means that enriched IL-22+ CD4+ T cells were placed in the upper chamber of Transwell culture system without physical contact with infected macrophages that were cultured in the lower chamber. Therefore, noncontact IL-22+ CD4+ T cells did not contact macrophages, but secretory IL-22 in the medium could trans-pass through the microholes and reach macrophages in the lower chamber. Contact means that IL-22+ CD4+ T cells and infected macrophages were cocultured in the lower chamber of Transwell culture system. Data in graph were mean values with error bars of SEM derived from four rhesus macaques. **p < 0.01. NS, no significant difference.

and lymphocytes from lung tissues containing TB granuloma, suggesting that membrane-bound IL-22 possesses long t½ and sustains even in TB inflammatory environments. Notably, membrane-bound IL-22 cytokine molecules are engaged as ~100–200 nm nanoclusters or ~300–600 nm high-density nanodomains on outer membrane of CD4+ T effector cells that mature in vivo. It is also noteworthy that IL-22 nanoclusters or nanodomains coexist with CD4 and CD3 nanoclusters or nanodomains on membrane of IL-22+ T effector cells. T cells bearing membrane nanoclusters/nanodomains of IL-22, CD4, and CD3 appear to be highly activated effector cells during M. tuberculosis infection or in granulomas formation, because we and others show that occurrence of CD3 and CD4 nanoclusters or nanodomains is a structured engagement of T cell activation for efficient TCR interaction with APC and for sustaining T cell activation (38, 39). More importantly, such membrane IL-22 nanoclusters or nanodomains may confer high-affinity interaction with IL-22R on responsive cells. Thus, membrane-bound IL-22+ CD4+ T cells appear to be immunologically selected to exert prolonged and high-affinity effects on IL-22R–expressing cells in infections. Our results provided the first evidence, to our knowledge, that purified IL-22+ CD4+ T cells could mediate inhibition of intracellular M. tuberculosis replication in monocyte-derived macrophages. Th1, Th2, Th17, and T regulatory cells are four major CD4 subsets themselves has remained poorly defined, because it is impossible to isolate or purify each of these Th subsets for functional analyses. The membrane-bound IL-22 as surface marker allowed us to isolate IL-22+ Th22 cells and evaluate their antimicrobial function. Using this approach, we demonstrated that IL-22+ T cells themselves could inhibit...
intracellular *M. tuberculosis* in macrophages in the culture system. To our knowledge, this is also the first demonstration that a purified Th subset is able to directly mediate the inhibition of intracellular bacterium in vitro, although soluble IL-22 has been shown to limit *M. tuberculosis* growth (41). The membrane IL-22 bioactivity appeared to be supported by the fact that membrane-bound IL-22 in purified T cells could be detected for 2–3 d in cultures (data not shown), and that anti-IL-22 Ab incubation with purified IL-22*CD4+ T* cells from the beginning throughout the assay could reduce the effector cells’ ability to limit *M. tuberculosis* growth. The result from Transwell system-based experiments also suggests that membrane-bound IL-22 appears to contribute to the IL-22*CD4+ T* effector-mediated restriction of *M. tuberculosis* replication in macrophages as *M. tuberculosis* growth is inhibited by membrane-bound IL-22*CD4+ T* cells contacting with *M. tuberculosis*-infected macrophages, but not by those noncontacting IL-22*CD4+ T* cells or culture medium containing soluble IL-22 secreted by the effector cells (Fig. 5D). Membrane-bound IL-22 may act on IL-22R expressed in *M. tuberculosis*-infected macrophages, as our new studies demonstrate that whereas IL-22R is constitutively expressed in epithelial cells, IL-22R expression can also be induced by *M. tuberculosis* infection of macrophages (Fig. 5A, and D. Huang, G. Zeng, C. Chen, R. Wang, and Z.W. Chen, manuscript in preparation). This is also supported by our previous observation that TB induces up-regulation of IL-22R (transcript (IL-22Rβ [IL-10Rβ]) (20)). Membrane-bound IL-22 and IL-22R interaction could help to bring together a T effector cell and an infected macrophage as well as to transduce a signal for anti-bacilli effector function. Thus, our results are consistent with the previous findings that IL-22 can exert antimicrobial activity against extracellular or intracellular pathogens (14–17, 41). The recent human study suggests that soluble IL-22 can limit *M. tuberculosis* growth in macrophages through a mechanism of enhancing phagolysosomal fusion (41). However, our data cannot exclude the possibility that other cytokines or interacting molecules of the IL-22*CD4+ T* cells also contribute to or synergize the in vitro anti- *M. tuberculosis* effector function (42). Our previous observations support the hypothesis that IL-22* T* cells may function as either proinflammatory or antimicrobial elements in *M. tuberculosis* infection, depending upon the magnitude of bacterial burdens that elicit optimal or overreacting IL-22* T* effector cells (23). Now the finding from the current study raises the possibility to define the in vivo function of membrane-bound IL-22* T* cells or IL-22 cytokine during *M. tuberculosis* infection of nonhuman primates.

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Disclosures

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