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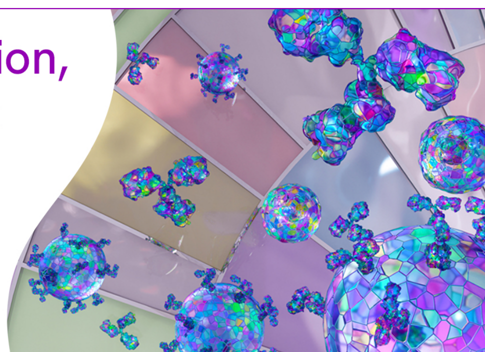

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Dominant Role for TL1A/DR3 Pathway in IL-12 plus IL-18-Induced IFN-γ Production by Peripheral Blood and Mucosal CCR9$^+$ T Lymphocytes[1]

Konstantinos A. Papadakis,[2] Daocheng Zhu, John L. Prehn, Carol Landers, Armine Avanesyan, Gina Lafkas, and Stephan R. Targan[2]

The TNF-like cytokine TL1A augments IFN-γ production by anti-CD3 plus anti-CD28 and IL-12/IL-18-stimulated peripheral blood (PB) T cells. However, only a small subset of PB T cells respond to TL1A stimulation with IFN-γ production. PB CCR9$^+$ T cells represent a small subset of circulating T cells with mucosal T cell characteristics and a Th1/Tr1 cytokine profile. In the current study, we show that TL1A enhanced IFN-γ production by TCR- or CD2/CD28-stimulated CCR9$^+$CD4$^+$ PB T cells. However, TL1A had the most pronounced effect on augmenting IFN-γ production by IL-12/IL-18-primed CCR9$^+$CD4$^+$ PB T cells. TL1A enhanced both the percentage and the mean fluorescence intensity of IFN-γ in CCR9$^+$CD4$^+$ T cells as assessed by intracellular cytokine staining. IL-12 plus IL-18 up-regulated DR3 expression in CCR9$^+$CD4$^+$ T cells but had negligible effect on CCR9$^-$CD4$^+$ T cells. CCR9$^+$CD4$^+$ T cells isolated from the small intestine showed a 37- to 105-fold enhancement of IFN-γ production when TL1A was added to the IL-12/IL18 cytokine combination. Cell membrane-expressed TL1A was preferentially expressed in CCR9$^+$CD4$^+$ PB T cells, and a blocking anti-TL1A mAb inhibited IFN-γ production by cytokine-primed CCR9$^+$CD4$^+$ T cells by ~50%. Our data show that the TL1A/DR3 pathway plays a dominant role in the ultimate level of cytokine-induced IFN-γ production by CCR9$^+$ mucosal and gut-homing PB T cells and could play an important role in Th1-mediated intestinal diseases, such as Crohn's disease, where increased expression of IL-12, IL-18, TL1A, and DR3 converge in the inflamed intestinal mucosa. *The Journal of Immunology,* **2005, 174: 4985–4990.**

Interferon-γ, a hallmark cytokine produced by Th1 cells and cells of the innate immune system, plays an important role in the elimination of intracellular pathogens and the pathogenesis of autoimmune/inflammatory diseases (1, 2). Several cytokines and co-stimulatory molecules have been shown to initiate or enhance Th1 differentiation of naive CD4$^+$ T cells (reviewed in Ref. 1). Differentiated Th1 cells produce IFN-γ upon TCR engagement, which is enhanced with co-stimulation with anti-CD28 Abs (3). Also differentiated Th1 cells can directly produce IFN-γ in response to IL-12/IL-18 in the absence of TCR engagement (4), and both act synergistically to enhance IFN-γ production in T cells and NK cells (5–7). Recent data have shown that IL-18 signaling induces the expression of growth arrest and DNA damage 45β (GADD45β),[3] which in turn activates p38 MAPK and induces IFN-γ transcription in CD4$^+$ T cells (8). IL-18-induced GADD45β expression is augmented severalfold by additional IL-12 signaling (8).

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[3] Abbreviations used in this paper: GADD45β, growth arrest and DNA damage 45β; SI, small intestine; mTL1A, cell membrane-associated TL1A; PB, peripheral blood.

TL1A, a TNF-like cytokine, has been recently identified to interact with the death domain-containing receptor DR3 and activates NF-κB in cells expressing endogenous DR3 (9). Expression of DR3 is restricted to lymphocytes and is up-regulated upon T cell activation. TL1A was shown to increase IL-2 responsiveness and enhance IFN-γ and GM-CSF release in anti-CD3- and anti-CD28-stimulated peripheral blood (PB) T cells (9). Recently, we have shown that TL1A or an agonistic anti-DR3 mAb enhances IFN-γ production by anti-CD3- or anti-CD2-stimulated PBL or lamina propria lymphocyte, independent of, but in synergy with, IL-12 and IL-18 (10). In addition, TL1A augments IFN-γ production by IL-12- plus IL-18-stimulated PB T and NK cells (11). Interestingly, only a small percentage of PB T cells responds to combined IL-12/IL-18 plus TL1A stimulation to produce IFN-γ (11).

CCR9$^+$ T lymphocytes represent a small subset of PB T cells that is involved in small intestinal immune and inflammatory responses (12). T lymphocytes expressing CCR9 are enriched in the lamina propria and intraepithelial lymphoid compartment of the small intestine (SI) (13, 14) and are enriched in the peripheral circulation of patients with SI Crohn's and celiac disease (15). Recently, CCR9$^+$CD4$^+$ T cells isolated from PB were reported to have mucosal T cell characteristics, namely an activation phenotype and responsiveness to CD2 activation, and a Th1 or Tr1 cytokine profile (12).

The aim of our study was to determine whether TL1A predominantly affects the CCR9$^+$ T cell subset of PBL for IFN-γ production in response to TCR or cytokine stimulation. We show that TL1A/DR3 interactions play a dominant role in the cytokine-induced IFN-γ production in CCR9$^+$CD4$^+$ T cells in the PB and the SI. Importantly, endogenous cell membrane-associated TL1A (mTL1A)/DR3 interactions partially mediate the IL12/IL-18-induced IFN-γ production in CCR9$^+$CD4$^+$ PB T cells, and therefore, this

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pathway plays a dominant role in the ultimate level of IFN-γ production produced by the combined IL-12/IL-18 stimulation.

Materials and Methods

Cytokines and Abs

Reagents generated at Human Genome Sciences (Rockville, MD) included recombinant human TL1A (aa 72–251), anti-DR3, and anti-TL1A mAb have been previously described (9, 16). These mAb were tested for specificity at Human Genome Sciences using cells monotransfected with a panel of TNF-family ligands or receptors. Recombinant IL-12 was obtained from Peprotech, and IL-18 was purchased from R&D Systems. These cytokines were used at concentrations ten times the approximate ED_{50} reported by the suppliers. Isotype- or species-specific control Abs were purchased from Jackson ImmunoResearch Laboratories. Anti-CD4 fluorochrome-conjugated mAb was obtained from Caltag Laboratories. Anti-IFN-γ PE-conjugated Ab and isotype mouse IgG1-PE for intracellular cytokine staining were purchased from BD Pharmingen. The mAb against human CCR9 (3C3, IgG2b) was a kind gift from Martin R. Hodge (Millennium Pharmaceuticals, Cambridge, MA). The anti-CD2 Abs (clones CB6 and GD10) were a generous gift from C. Benjamin (Biogen, Cambridge, MA).

Cell isolation, sorting, and FACS analysis

PBL were isolated from normal healthy volunteers by separation on Ficoll-Hypaque gradients. The cells were cultured in RPMI 1640 containing 2 mM glutamine and 25 mM HEPES buffer (Mediatech), supplemented with 10% heat-inactivated FBS (Atlanta Biologicals), 50 μg/ml gentamicin (Omega Scientific), and 0.25 μg/ml amphotericin B (Gemini Bioproducts). Isolation of SI mucosal lymphocytes was performed as previously described (14). Cells isolated from the PB or SI were stained with anti-CCR9 mAb (3C3) followed by a secondary PE- or tricolor-conjugated goat anti-mouse IgG2b mAb (Caltag Laboratories). The cells were washed and incubated with mouse IgG for 15 min and subsequently stained with FITC-conjugated anti-CD4 mAb. After staining, the cells were sorted using FACSVantage (BD Biosciences) to isolate CD4$^+$CCR9$^+$ and CD4$^+$CCR9$^-$ cells (purity ~98%). In some experiments, PBL were stained with FITC-conjugated anti-CD4, PE-conjugated anti-CD45RO and anti-CCR9-TC and sorted into CD4$^+$CD45RO$^+$CCR9$^+$ and CD4$^+$CD45RO$^+$CCR9$^-$ cells. For staining of cell surface, DR3 and TL1A, 5 × 10 (5) freshly isolated or cultured PBL were washed twice with PBS supplemented with 0.1% BSA and 0.1% azide and resuspended in 100 μl of 2% FCS/PBS for 15 min. The cells were incubated with the anti-CCR9 mAb 3C3 and anti-TL1A or anti-DR3 mAbs for 30 min on ice, washed with PBS/BSA/azide and incubated with a secondary goat anti-mouse IgG2b-TC and anti-mouse IgG2a-PE (for mTL1A staining) or anti-mouse IgG1-PE (for DR3 staining) for 30 min on ice. The cells were washed again with PBS/BSA/azide and incubated with mouse IgG for 15 min followed by a FITC-conjugated mAb for CD4. After washing twice, cells were resuspended in 400 μl of 1% paraformaldehyde in PBS and analyzed by FACS (BD Biosciences); 3 × 10^4 events were routinely collected and analyzed using CELLQuest software (BD Immunocytometry Systems).

Cell stimulation and blocking experiments

Sorted CD4$^+$CCR9$^+$ and CD4$^+$CCR9$^-$ T cells were stimulated with plate-bound anti-CD3 (American Type Culture Collection (ATCC); OKT3, 1 μg/ml) ± anti-CD28 (clone 9.3, 2 μg/ml) or with anti-CD2 (2 μg/ml) ± anti-CD28 (2 μg/ml) and with or without TL1A for 72 h in 96-well flat-bottom plates. In other experiments, sorted CD4$^+$CCR9$^+$ and CD4$^+$CCR9$^-$ T cells were stimulated with IL-12 (2 ng/ml) and IL-18 (50 ng/ml) with or without TL1A or an agonistic anti-DR3 mAb (F05) for 72 h in 96-well round-bottom plates. In some experiments, sorted CD4$^+$CD45RO$^+$CCR9$^+$ and CD4$^+$CD45RO$^+$CCR9$^-$ cells were also stimulated with IL-12 (2 ng/ml) and IL-18 (50 ng/ml) with or without TL1A for 72 h in 96-well round-bottom plates. Neither IL-12 nor IL-18 alone enhanced IFN-γ secretion by TL1A-stimulated CD4$^+$CCR9$^+$ and CD4$^+$CCR9$^-$ T cells (data not shown). Also preliminary experiments showed that the optimal duration of stimulation for T cell enhancement of IFN-γ secretion was 72 h (17).

For blocking experiments, FACS-sorted CD4$^+$CCR9$^+$ T cells were incubated with IL-12/IL-18 and anti-TL1A (10 μg/ml) or an isotype control IgG2a mAb for 72 h in 96-well round-bottom plates. The supernatants were assayed for IFN-γ by ELISA.

Detection of IFN-γ by ELISA and intracellular staining

IFN-γ was quantitated in culture supernatants by amplified sandwich ELISA as previously reported (12). Briefly, 96-well microtiter plates were coated overnight with anti-IFN-γ mAb (BD Pharmingen). After blocking in PBS-BSA, diluted standards (rhIFN-γ; R&D Systems) and samples were added for 24–72 h and detected by a second anti-IFN-γ biotinylated mAb (BD Pharmingen) for 2 h. After washing, this biotinylated mAb was detected by streptavidin-alkaline phosphatase (Jackson ImmunoResearch Laboratories) for 30 min followed by four phosphate-free washes. Substrate (0.2 mM NADPH; Sigma-Aldrich) was added for 30 min, then the NADH signal was amplified using 2-propanol (3%) with iodonitrotetrazoliun violet (1 mM), alcohol dehydrogenase (75 μg/ml), and diaphorase (50 μg/ml; the last three were obtained from Sigma-Aldrich), and plates were read at 490 nm on an Emax plate reader (Molecular Devices). Sample concentration was calculated from a standard curve generated by our own software (R. Deem).

For cytokine detection at the single cell level, sorted CD4$^+$ T cell subsets were stimulated with IL-12 (2 ng/ml) and IL-18 (50 ng/ml) with or without TL1A for 72 h. Brefeldin A (10 μg/ml) was added to the culture for the last 5 h of stimulation to block cytokine secretion. Cells were fixed and permeabilized using cytofix/cytoperm solution (Caltag Laboratories), stained with PE-conjugated control Ab or mAb to IFN-γ and analyzed by FACS.

Results

Effect of TL1A on TCR or CD2-stimulated IFN-γ production by CCR9$^+$ and CCR9$^-$CD4$^+$ T cells

We previously reported that CCR9$^+$CD4$^+$ T cells from PB have mucosal T cell characteristics, in that they exhibit an activated phenotype and respond to CD2 stimulation, a hallmark activation pathway for mucosal T cells. Compared with CCR9$^-$, CCR9$^+$CD4$^+$ T cells produce more IFN-γ in response to TCR or anti-CD2 stimulation (12). Moreover, TL1A a newly described TNF-like molecule, enhances IFN-γ production by a small subset of PB T cells when stimulated with anti-CD3 and anti-CD28 mAbs or with IL-12 plus IL-18 (11). Since CCR9$^+$CD4$^+$ T cells represent only a small subset of PB CD4$^+$ T cells (~2–4%) (12, 18), we examined whether TL1A enhances IFN-γ production selectively in this subset. As shown in Fig. 1*A*, TL1A enhanced IFN-γ production by anti-CD3- plus anti-CD28-stimulated CCR9$^+$CD4$^+$ T cells by ~2-fold. Similar fold enhancement of IFN-γ production was seen in anti-CD3- plus anti-CD28-stimulated CCR9$^-$CD4$^+$ T cells, although the absolute amount of IFN-γ produced was lower in CCR9$^-$ compared with CCR9$^+$CD4$^+$ T cells (Fig. 1*A* and Ref. 12). The IFN-γ-enhancing effect of TL1A in both cell subsets required CD28 costimulation consistent with the previous observation in unfractionated PB T cells (9). Since CCR9$^+$CD4$^+$ T cells respond to CD2 stimulation with proliferation and cytokine production, we next examined whether TL1A also costimulates this

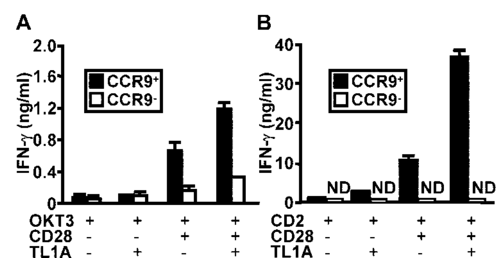

FIGURE 1. Effect of TL1A on IFN-γ production by anti-CD3- or anti-CD2- plus anti-CD28-stimulated CCR9$^+$ and CCR9$^-$CD4$^+$ PB T cells. FACS-sorted CCR9$^+$ and CCR9$^-$CD4$^+$ PB T cells were activated with plate-bound anti-CD3 (OKT3, 1 μg/ml) ± anti-CD28 (2 μg/ml) (*A*) or anti-CD2 (2 μg/ml) ± anti-CD28 (2 μg/ml) with or without TL1A (100 ng/ml) in 96-well flat-bottom plates for 72 h (*B*). IFN-γ content was analyzed in culture supernatants by ELISA. Representative of three experiments with similar results is shown. ND, Not detected.

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cell subset to enhance IFN-γ production through the CD2 pathway. Fig. 1*B* shows that TL1A (100 ng/ml) enhanced the secretion of IFN-γ by anti-CD2- plus anti-CD28-stimulated CCR9$^+$CD4$^+$ T cells by ~3-fold. Similarly, the IFN-γ-enhancing effect of TL1A required CD28 costimulation. As expected CCR9$^-$CD4$^+$ T cells did not secrete any detectable IFN-γ in response to anti-CD2 ± anti-CD28 stimulation as previously described (12) with or without TL1A (Fig. 1*B*). TL1A when used alone had no effect on IFN-γ secretion by CCR9$^+$ or CCR9$^-$CD4$^+$ T cells (data not shown).

TL1A enhances cytokine-induced IFN-γ production by CCR9$^+$CD4$^+$ T cells

Since Th1 cells can produce IFN-γ in response to combined cytokine stimulation with IL-12 and IL-18 and CCR9$^+$CD4$^+$ T cells contain differentiated Th1 cells, we next examined whether this cytokine combination could enhance IFN-γ production by CCR9$^+$CD4$^+$ T cells and if TL1A had an enhancing effect. Interestingly, the fold enhancement of IFN-γ secretion by TL1A was higher when CCR9$^+$CD4$^+$ T cells were stimulated with IL-12 plus IL-18 rather than through the CD3 or CD2 pathway and costimulated with anti-CD28. The fold change in IFN-γ secretion by the addition of TL1A on cytokine-induced IFN-γ production ranged from 3.6 to 55 in four different donors examined (Table I). The addition of an agonistic anti-DR3 mAb (clone F05) had an enhancing effect similar to TL1A on IFN-γ production by cytokine-stimulated CCR9$^+$CD4$^+$ PB T cells (data not shown), indicating that the TL1A effect on CCR9$^+$ T cells is mediated through the DR3 receptor. TL1A markedly enhanced IFN-γ secretion by IL-12/IL-18-stimulated CCR9$^+$CD4$^+$ T cells in a dose-dependent manner (Fig. 2*A*), whereas it had a minimal effect on CCR9$^-$CD4$^+$ T cells. To further characterize the sensitivity of CCR9$^+$CD4$^+$ T cells on the co-stimulatory effect of TL1A on cytokine-induced IFN-γ production, we used lower concentrations of TL1A ranging from 0.1 to 10 ng/ml. As shown in Fig. 2*B*, TL1A, at much lower concentrations, dose dependently increased the secretion of IFN-γ by cytokine-stimulated CCR9$^+$CD4$^+$ T cells. TL1A, at a concentration as low as 500 pg/ml, enhanced the cytokine-induced IFN-γ production in CCR9$^+$CD4$^+$ PB T cells by an average of 10-fold (Fig. 2*B*). TL1A had a minimal effect on cytokine-induced IFN-γ production in CCR9$^-$CD4$^+$ T cells. Incubation of CCR9$^+$ or CCR9$^-$CD4$^+$ T cells with IL-12 or IL-18 singly did not enhance TL1A or anti-DR3-induced IFN-γ production (data not shown).

To determine whether the enhancing effect of TL1A on cytokine-induced IFN-γ production by CCR9$^+$ T cells is due to the higher frequency of memory T cells in this subset compared with CCR9$^-$ T cells, we analyzed the effect of TL1A on sorted memory cell populations. As shown in Fig. 3*A*, TL1A stimulation enhanced IFN-γ production by cytokine-stimulated memory CCR9$^+$ and CCR9$^-$CD4$^+$ T cells. Although the fold enhancement of IFN-γ was similar between memory CCR9$^+$ and CCR9$^-$ T cells (5- vs 4.5-fold, $n = 3$), memory CCR9$^+$ T cells produced significantly more IFN-γ in response to IL-12/IL-18 and TL1A stimulation (Fig. 3*A*). Nevertheless, a small subset of non-gut-homing (CCR9$^-$) memory CD4$^+$ PB T cells appears also to respond to TL1A stimulation.

When we analyzed the production of IFN-γ at the single cell level, we found that both the percentage and the mean fluorescence intensity of IFN-γ-producing cells was increased in CCR9$^+$, but not CCR9$^-$CD4$^+$ T cells, when stimulated with IL-12/IL-18 plus TL1A compared with IL-12/IL-18 stimulation alone (Fig. 3*B*). These data show that TL1A enhances both the number of IFN-γ-secreting cells and the amount of IFN-γ produced per cell in cytokine-stimulated CCR9$^+$CD4$^+$ T cells.

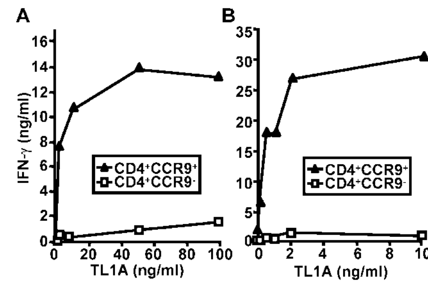

FIGURE 2. TL1A dose dependently enhances IFN-γ production by IL-12 and IL-18-stimulated CCR9$^+$CD4$^+$ T cells. FACS-sorted PB CCR9$^+$ and CCR9$^-$CD4$^+$ T cells were incubated with IL-12 (2 ng/ml) and IL-18 (50 ng/ml) and with or without higher (*A*) or lower (*B*) concentrations of TL1A for 72 h. IFN-γ content was analyzed in culture supernatants by ELISA. Representative of three experiments (*A*) and two experiments (*B*) with similar results is shown.

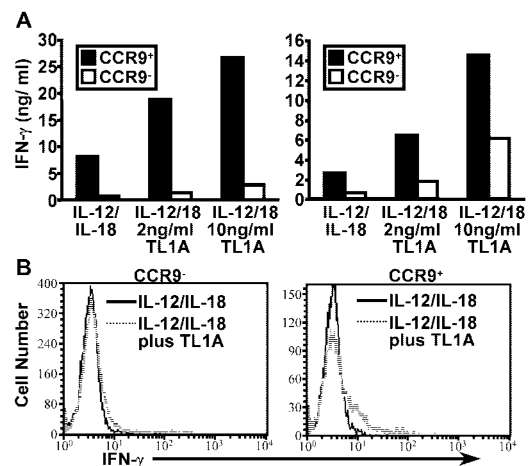

FIGURE 3. *A*, TL1A enhances IFN-γ production by cytokine-stimulated CCR9$^+$ and CCR9$^-$ PB memory T cells. PBL were sorted into CCR9$^+$CD4$^+$ or CCR9$^-$CD4$^+$ T cells (*left panel*) or CCR9$^+$CD45RO$^+$CD4$^+$ or CCR9$^-$CD45RO$^+$CD4$^+$ T cells (*right panel*) from the same donor and stimulated with IL-12 (2 ng/ml), IL-18 (50 ng/ml) with or without TL1A (2 or 10 ng/ml) for 72 h. IFN-γ content was analyzed in culture supernatants by ELISA. Representative of three experiments with similar results is shown. *B*, TL1A augments the number of IFN-γ-producing cells and the amount of IFN-γ produced per cell in CCR9$^+$ T cells. FACS-sorted CCR9$^-$ (*left histogram*) and CCR9$^+$CD4$^+$ PB T cells (*right histogram*) were stimulated with IL-12/IL-18 alone (solid lines) or with TL1A (dotted lines) and stained for intracellular IFN-γ as described in *Materials and Methods*. The experiment was performed three times with similar results.

Table I. *TL1A enhances IFN-γ production by cytokine-stimulated PB CCR9$^+$CD4$^+$ T cells*[a]

PB CCR9$^+$CD4$^+$	IL-12/IL-18-Induced IFN-γ (ng/ml)	IL-12/IL-18 plus TL1A-Induced IFN-γ (ng/ml)	Fold Enhancement
Donor 1	1.7	21.7	13
Donor 2	23	82.2	3.6
Donor 3	23.7	115	4.8
Donor 4	0.195	10.7	55

[a] FACS-sorted PB CCR9$^+$CD4$^+$ T cells were stimulated with IL-12 (2 ng/ml) plus IL-18 (50 ng/ml) with or without TL1A (100 ng/ml) for 72 h. Culture supernatants were analyzed for IFN-γ content by ELISA.

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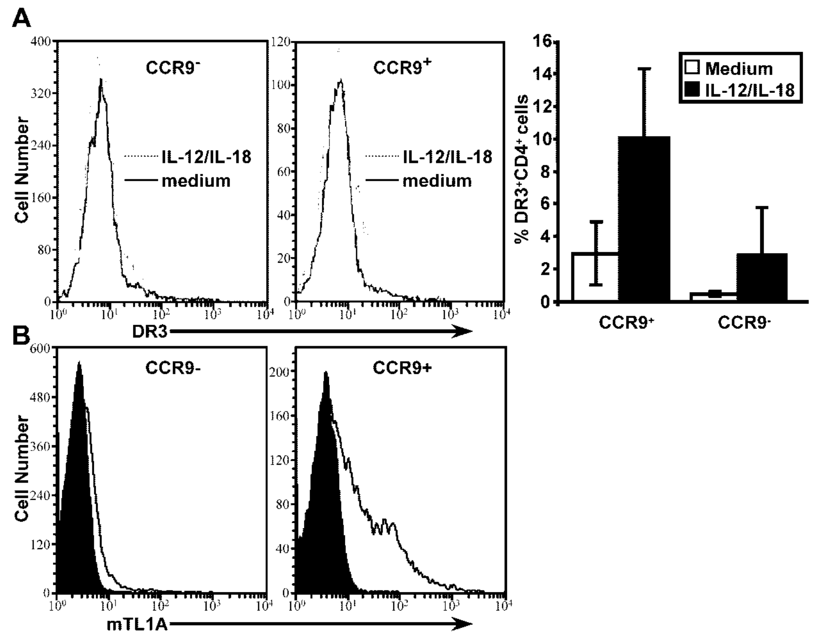

FIGURE 4. *A,* DR3 expression is increased in CCR9$^+$CD4$^+$ T cells following stimulation with IL-12 plus IL-18. FACS-sorted CCR9$^-$ (*left histograms*) and CCR9$^+$CD4$^+$ PB T cells (*right histograms*) were incubated with medium (solid lines) or IL-12 (2 ng/ml) plus IL-18 (50 ng/ml) for 48 h (dotted lines), incubated with anti-DR3 and a secondary anti-IgG1-PE-conjugated secondary Ab, and analyzed by FACS. The *right panel* shows the percentage of DR3-expressing CCR9$^+$ or CCR9$^-$CD4$^+$ T cells with medium alone or following stimulation with IL-12/IL-18 ($n = 5$). *B,* mTL1A is primarily expressed by CCR9$^+$CD4$^+$ T cells. FACS-sorted CCR9$^+$ and CCR9$^-$CD4$^+$ PB T cells were stained with anti-TL1A mAb followed by a secondary anti-IgG2a-PE-conjugated Ab and analyzed by flow cytometry. Representative experiment of five with similar results is shown. Shaded histogram indicates staining with an isotype control Ab.

The enhancing effect of TL1A on cytokine-induced IFN-γ production by CCR9$^+$CD4$^+$ T cells could be mediated through differential regulation of DR3 expression and/or postreceptor signaling in these cells in response to combined IL-12 plus IL-18 stimulation. Previous studies have shown that DR3 is not expressed in freshly isolated PB CD4$^+$ T cells but is up-regulated following TCR stimulation (19). Therefore, we analyzed the expression of DR3 in CCR9$^+$ and CCR9$^-$CD4$^+$ T cells following stimulation with IL-12/IL-18. As shown in Fig. 4*A*, the combined IL-12/IL-18 stimulation up-regulated DR3 expression in CD4$^+$CCR9$^+$ T cells but had a negligible effect on CD4$^+$CCR9$^-$ T cells. The percentage of CCR9$^+$CD4$^+$ T cells increased from 2.9 ± 2% with medium alone to 10 ± 4.2% with IL-12/IL-18 stimulation ($n = 5$; $p < 0.001$), whereas CCR9$^-$CD4$^+$ T cells increased from 0.4 ± 0.16% with medium alone to 2.9 ± 2.9% with IL-12/IL-18 stimulation ($n = 5$; $p = 0.01$) (Fig. 4*A*, *right panel*).

We have previously shown that CCR9$^+$ T cells from PB have an activated phenotype and express several TNF-like molecules. Therefore, we examined whether mTL1A, which is up-regulated upon TCR stimulation in a small subset of PB T cells (10), is preferentially expressed among CD4$^+$CCR9$^+$ T cells. Interestingly, mTL1A was almost exclusively expressed in freshly isolated or FACS-sorted CCR9$^+$CD4$^+$ T cells (Fig. 4*B*). The percentage of CCR9$^+$CD4$^+$ T cells that expressed mTL1A was 25% (range, 11–47%; $n = 5$) compared with 2.5% (range, <0.1–7%; $n = 5$; $p = 0.02$) of CCR9$^-$CD4$^+$ T cells. IL-12/IL-18 stimulation did not affect mTL1A expression in either cell subset (data not shown).

Cytokine-induced IFN-γ production in CCR9$^+$CD4$^+$ T cells is partially mediated through TL1A/DR3 interactions

Since IL-12/IL-18 enhanced DR3 expression and mTL1A is expressed almost exclusively in CCR9$^+$CD4$^+$ T cells, we reasoned that IL-12/IL-18-induced IFN-γ production in this cell subset could be mediated at least partially through endogenous TL1A/DR3 interactions. To this end, anti-TL1A, which binds to mTL1A and inhibits its interaction with DR3, partially inhibited cytokine-induced IFN-γ in CCR9$^+$CD4$^+$ T cells (Fig. 5). The average inhibition of IFN-γ secretion by anti-TL1A mAb treatment of cytokine-stimulated CCR9$^+$CD4$^+$ T cells was 46% (range, 29–66%; $n = 5$) compared with an isotype control mAb. Therefore, mTL1A/DR3 interactions partially mediate IFN-γ production by IL-12/IL-18-stimulated PB CCR9$^+$CD4$^+$ T cells.

TL1A enhances cytokine-induced IFN-γ production by CD4$^+$CCR9$^+$ small intestinal lymphocytes

Since CCR9$^+$ T cells localize to the SI (13, 14), we examined whether TL1A could enhance IFN-γ production by purified SI CCR9$^+$CD4$^+$ T cells. TL1A markedly enhanced IFN-γ production by cytokine-stimulated CCR9$^+$CD4$^+$ T cells isolated from the SI. The fold increase in IFN-γ production ranged from 37 to 107 in three separate experiments (Table II). Taken together, our data show that the TL1A/DR3 costimulatory pathway markedly enhances IFN-γ production by IL-12/IL-18-stimulated peripheral and mucosal CCR9$^+$CD4$^+$ T cells.

Discussion

In the current study, we show that TL1A/DR3 interactions play an important role in TCR but mainly in cytokine-induced IFN-γ production by PB and mucosal CCR9$^+$CD4$^+$ T cells and, therefore,

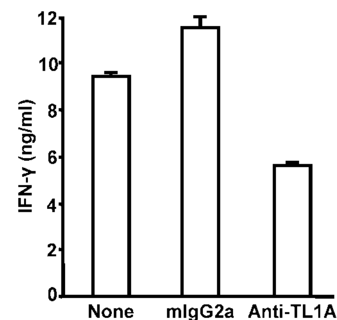

FIGURE 5. TL1A/DR3 interactions partially mediate IL-12 plus IL-18-induced IFN-γ production in CCR9$^+$CD4$^+$ PB T cells. FACS-sorted CCR9$^+$CD4$^+$ T cells were incubated with IL-12 (2 ng/ml) plus IL-18 (50 ng/ml) in 96-well round-bottom plates with or without a blocking anti-TL1A (10 μg/ml) or an isotype control Ab for 72 h. Culture supernatants were assayed for IFN-γ content by ELISA. Representative of five experiments with similar results is shown.

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Table II. *TL1A enhances IFN-γ production by cytokine-stimulated SI $CCR9^+CD4^+$ T cells*[a]

SI $CCR9^-CD4^-$	IL-12/IL-18-Induced IFN-γ (ng/ml)	IL-12/IL-18 plus TL1A-Induced IFN-γ (ng/ml)	Fold Enhancement
Donor 1	0.347	39.3	105
Donor 2	2	93.74	47
Donor 3	2.2	82	37

[a] FACS-sorted SI $CCR9^+CD4^+$ T cells were stimulated with IL-12 (2 ng/ml) plus IL-18 (50 ng/ml) with or without TL1A (100 ng/ml) for 72 h. Culture supernatants were analyzed for IFN-γ content by ELISA.

may determine the ultimate level of IFN-γ production by effector mucosal or mucosal-trafficking PB T cells. Importantly, T-T interactions through mTL1A and DR3 could partially mediate cytokine-induced IFN-γ production by $CCR9^+CD4^+$ T cells. Since IL-12, IL-18, and TL1A/DR3 expression are all increased in inflamed intestinal tissues in Crohn's disease, this pathway of IFN-γ production by effector mucosal T cells may be critical for disease pathogenesis.

TL1A, a recently identified TNF-like molecule, is induced in endothelial cells by proinflammatory cytokines and enhances IL-2 responsiveness and IFN-γ and GM-CSF secretion by anti-CD3- and anti-CD28-stimulated PB T cells (9). TL1A also enhanced TCR-induced IFN-γ production in both PB and mucosal T cells independent of, but in synergy with, IL-12 and IL-18 (10). More recently, we reported that TL1A also enhances IL-12/IL-18-induced IFN-γ production in PB T and NK cells (11). Interestingly, only a small percentage (~1%) of PB T cells respond to combined IL-12/IL-18 plus TL1A stimulation with IFN-γ production and this could represent a subset of circulating $CCR9^+$ T cells (12). $CCR9^+CD4^+$ T cells represent ~2–4% of PB $CD4^+$ T cells and contain differentiated Th1 cells. Therefore, we examined the effect of TL1A on both cytokine- and TCR-induced IFN-γ production in this T cell subset. Interestingly, we found that the most pronounced enhancement of IFN-γ production by $CCR9^+CD4^+$ T cells in response to TL1A was observed when the cells were stimulated with cytokines rather than through their TCR/CD3 complex or the CD2 pathway. Importantly, we also found that $CCR9^+$ T cells appear to be extremely sensitive to TL1A stimulation because even 100 pg of TL1A enhanced IFN-γ production of cytokine-stimulated cells by severalfold (Fig. 2). It was recently reported that mTL1A is also induced in a small subset of PB T cells following in vitro TCR activation (20). In the current study, we show that $CCR9^+CD4^+$ T cells constitutively express mTL1A, which is consistent with their activated phenotype (12). This cell subset from PB also constitutively expresses several TNF-like molecules, including CD40L, OX40, and CTLA-4. Several of the TNF-like molecules have been reported to enhance Th1 immune responses and could be involved in the pathogenesis of several autoimmune diseases (21–23). The constitutive expression of mTL1A on a subset of $CCR9^+CD4^+$ T cells and the up-regulation of DR3 expression by IL-12/IL-18 stimulation indicated that endogenous mTL1A/DR3 interaction could partially mediate cytokine-induced IFN-γ production. Indeed, the addition of a blocking anti-TL1A Ab partially inhibited cytokine-induced IFN-γ production in $CCR9^+CD4^+$ T cells. These data provide compelling evidence of the prominent role for mTL1A/DR3 interactions in the augmentation of cytokine-induced IFN-γ production in PB and mucosal $CCR9^+CD4^+$ T cells. TL1A increased both the percentage and the mean fluorescence intensity of IFN-γ-secreting $CCR9^+CD4^+$ T cells. These data indicate that one of the effects of TL1A/DR3 interaction is to increase the production of IFN-γ on a per cell basis. Since IL-12/IL-18-induced IFN-γ is mediated by several signaling molecules, including GADD45β, NF-κB, and p38 MAPK, the addition of TL1A could synergize for the induction of IFN-γ through the activation of NF-κB and p38 MAPK (8, 24). Recently, TL1A was reported to activate NF-κB and p38 MAPK in the TF1 erythroleukemia cell line (16). Further studies are required to determine the molecular mechanisms by which TL1A enhances IL-12/IL-18-induced IFN-γ production in T cells.

Collectively, our data show that TL1A profoundly enhances the production of IFN-γ by cytokine-stimulated $CCR9^+CD4^+$ PB and mucosal T cells and that IL-12/IL-18-induced IFN-γ production in this T cell subset is partially mediated through mTL1A/DR3 and T-T cell interactions. Both IL-12 and IL-18 are up-regulated in the intestinal mucosa in Th1 inflammatory diseases such as Crohn's disease (25–27), and it has been proposed that cytokine-induced IFN-γ production by T cells may play a key role in the pathogenesis of this disease (28, 29). Recent studies have also shown that mTL1A is expressed by macrophages and a subset of mucosal T cells in inflamed intestinal mucosa in Crohn's disease (30) and following T cell activation in vitro (10). DR3 expression, which is restricted on T cells, was also increased in inflamed mucosa (20, 30). The profound effect of TL1A on cytokine-induced IFN-γ production in $CCR9^+$ PB and mucosal T cells suggests that macrophage-T or T-T interactions through mTL1A-DR3 pathway could be important for Crohn's disease pathogenesis. IL-12/IL-18 and mTL1A/DR3 may play a dominant role in the ultimate level of IFN-γ produced in the inflamed intestinal mucosa, where their expression converges. Therefore, blocking TL1A/DR3 interactions may represent a novel therapeutic target for the treatment of Crohn's disease.

Disclosures

The authors have no financial conflict of interest.

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