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TLR3 Ligand Polyinosinic: Polycytidylic Acid Induces IL-17A and IL-21 Synthesis in Human Th Cells

Christian K. Holm,* Charlotte C. Petersen,* Malene Hvid,* Line Petersen,* Søren R. Paludan,* Bent Deleuran,*† and Marianne Hokland*

TLR3 and TLR9 recognize the pathogen-associated microbial patterns dsRNA and unmethylated DNA, respectively. The recent discovery that these receptors also recognize endogenous ligands from necrotic material has drawn increased attention to their involvement in autoimmunity. Th cell cytokines IL-17A and IL-21 have been assigned pivotal roles in the regulation of such autoimmune diseases. IL-17A is the hallmark cytokine of the recently discovered proinflammatory Th cell subset T_N17. By contrast, the expression of IL-21 does not seem to be limited to a single distinct Th cell subset. We investigated the expression of IL-17A and IL-21 in human CD4^+ T cells in response to stimulation with the TLR3 ligand polyinosinic:polycytidylic acid (poly(I:C)) and the TLR9 ligand CpG. We discovered that poly(I:C) induced synthesis of both IL-17A and IL-21. Moreover, we found that poly(I:C) was able to drive the differentiation of naive Th cells into an IL-21 but not into an IL-17A-producing phenotype and did this without affecting the levels of transcription factors T-bet, GATA-3, or retinoic acid receptor-related orphan receptor C. Finally, we found that the IL-21-producing cells that were differentiated in response to poly(I:C) expressed the chemokine receptor CXCR3, which is important in the recruitment of T cells into inflamed joints in rheumatoid arthritis. This is the first report to show that the TLR3 ligand poly(I:C) can directly induce the synthesis of IL-17A and IL-21 and drive differentiation of human naive CD4^+ T cells.

Members of the TLR family have recently emerged as potential regulators of chronic inflammation in autoimmune diseases (1). This is motivated by the ability of TLRs to respond to pathogen-associated microbial patterns by inducing secretion of inflammatory cytokines, which are known to dominate chronic inflammation in autoimmunity (2–4). Each TLR member operates through a selection of distinct MyD88-dependent or -independent molecular pathways. These pathways are known to induce transcription through activation of the NF-κB complex, the stress-activated protein kinases (SAPK), and/or through activation of members of the IFN response factor family (IRFs) (5–7). Recent evidence identifies TLR3 and TLR9 as particularly important in chronic inflammation. In one study, TLR3 expression was increased in the synovial membrane from inflamed joints in rheumatoid arthritis (RA) and was shown to react to endogenous RNA from necrotic cells of these joints (8). In another study, TLR3 expression in inflammatory joints correlated with the presence of IL-1β, IL-18, and IFN-α (9). These data suggest that TLR3 ligands are accessible in chronically inflamed tissues and that the activation of the TLR3 pathways contributes to this inflammation in autoimmune diseases. The potential of TLR9 to regulate disease-associated factors in autoimmunity has been shown on several occasions. TLR9 mRNA is increased in PBMCs from patients with systemic lupus erythematous (SLE), and the production of rheumatoid factor by autoimmune B cells is triggered by chromatin-IgG complexes via TLR9 (10–12). The increased TLR9 expression in SLE correlates with severity of disease and to levels of anti-dsDNA Abs (12). The current knowledge of TLRs and their involvement in autoimmunity is primarily based on the study of B cells, macrophages, and fibroblasts. By contrast, only limited attention has been given to expression of TLRs on T cells. T cells infiltrate inflamed tissues in autoimmune diseases (13, 14) and since these cells also express functional TLRs (15), they clearly have the potential to contribute to the regulation of autoimmunity through this route. In support of this view, recent reports have showed that T cells activated directly through a selection of TLRs promote inflammation in animal models for autoimmune diseases (2, 15–17). However, whether TLR-dependent pathways can induce T cells to produce cytokines that are related to chronic inflammation is largely unexplored territory.

In the past decade, numerous reports have shown that Th cell-derived cytokines IL-17A and IL-21 are associated with autoimmune diseases (18–27). IL-17A is the hallmark cytokine of the Th cell subset T_N17 (18, 19, 28) and induces inflammation by stimulating epithelial and endothelial cells to secrete cytokines such as IL-6, IL-8, G-CSF, and PGE_2 (29, 30). In mice, the differentiation of naive Th cells into the T_N17 subset has been well documented (24, 28, 31, 32) while the differentiation in humans still remains to be fully characterized. However, naive human Th cells were shown to the differentiate into the T_N17 subset in vitro, in response to treatment with either IL-1β and IL-23 (33) or by IL-6, IL-21, and TGF-β (27, 34). Although IL-21 is produced by Th cells and
regulates the effector functions of both Tp1, Tp2, and Tp17 subsets, it is unresolved to which of these subsets, if any, IL-21 production belongs (24, 32, 35–37). The proposed segregation of IL-21-producing Th cells is currently based on a study showing that IL-6 increases the production of IL-21 without affecting the production of IL-17A, IL-4, or IFN-γ (38). The IL-21R is expressed on endothelia, fibroblasts, and leukocytes. Of particular interest to the area of autoimmunity is that synovial fibroblasts and mononuclear cells from RA patients (23) have increased expression of IL-21R and increased production of TNF-α in response to IL-21 in comparison to cells from healthy controls (25). The effects of IL-21 on leukocytes are pleiotropic. On one hand, IL-21 is essential for Ig production and plasma cell differentiation, but on the other hand IL-21 promotes apoptosis in B cells receiving inadequate activation (e.g., LPS alone) (39–42). Such antagonizing effects are also observed in CD8+ T cells (43). In addition, IL-21 has lately been shown to be a central player in the differentiation of CD4+ T cells and makes these cells nonresponsive to suppression by T regulatory cells (27, 32, 34, 44). Although much is known about the effects of IL-17A and IL-21, very little is known about how synthesis of these cytokines can be initiated, especially through pathways that are potentially activated in autoimmunity.

To investigate whether direct activation of human Th cells through TLR3 or TLR9 could affect the production of IL-17A or IL-21, we isolated human Th cells from healthy donors and stimulated these in the presence of the TLR3 ligand poly(I:C) or the TLR9 ligand CpG. We found that poly(I:C) but not CpG induced the synthesis of both IL-17A and IL-21 in human CD4+ T cells and that this induction depended on NF-kB and SAPK pathways. In addition, we found that polyinosinic:polycytidylic acid (poly(I:C)) induced differentiation of naive Th cells into an IL-21 but not into an IL-17A-producing phenotype. This phenotype expressed the RA-associated chemokine receptor CXCR3 but not the skin-associated CCR4. Together, these data point to an important role of TLR3 in the regulation of CD4+ T cells in autoimmunity.

Materials and Methods

Isolation and culture of cell subsets

Human PBMC were isolated from healthy donors using Ficoll-Paque (GE Healthcare). Sampling was performed according to protocols approved by the Ethics Committee for the County of Aarhus. Isolation of Th cells, memory Th cells, and naive Th cells was performed using negative isolation kits and the Midi-MACS isolation system with LD magnetic separation columns (Miltenyi Biotec). These kits included depleting Abs against CD8, CD14, CD16, CD19, CD36, CD56, CD123, TCR-γδ, and CD255a for crude CD4+ T and memory CD4+ T cell isolations and CD45RO, CD8, CD14, CD15, CD16, CD19, CD25, CD34, CD56, CD126, anti-CCR7, and CD235a for isolation of naive CD4+ T cells. Subsequent analysis of cell culture revealed purities of >97% with 2% contamination by CD8+ T cells. Contamination by APCs or NK cells was determined by being <0.1% as measured by flow cytometry. Cells were cultured in RPMI 1640 supplemented with 10% FCS (v/v), glutamine, and antibiotics. For activation of T cells, culture trays were precoated with 1 μg/ml in sterile PBS. TLR ligands were added at the initiation of cell culture. IL-23 was generated whole cell fractions labeled with anti-TLR3 and anti-TLR9 for analysis by flow cytometry. Whole cell fractions were analyzed within 24 h on a Cytomics FC500 flow cytometer (Beckman Coulter). Flow data were analyzed using FlowJo software (Tree Star). Cell sorting of CD4+ T cells was performed on a FACS Aria (BD Biosciences) by labeling non-CD4+ T cells with FITC-conjugated anti-CD4, anti-CD16, anti-CD19, and anti-CD23 (DakoCytomation). Cytokine concentrations in cell supernatants were measured using Ready Set Go ELISA kits (eBioscience).

Western blotting

Cells for Western blotting were lysed using 50 μM Tris-HCl, 10 μM EDTA, and 1% SDS (w/v) with the addition of complete mini protease inhibitors (Roche). SDS-PAGE was performed with cell lysates and nuclear extracts using Western blotting. SDS-PAGE was performed with cell lysates and nuclear extracts using Western blotting. TLR ligands were added at the initiation of cell culture. IL-23 was generated whole cell fractions labeled with anti-TLR3 and anti-TLR9 for analysis by flow cytometry. Whole cell fractions were analyzed within 24 h on a Cytomics FC500 flow cytometer (Beckman Coulter). Flow data were analyzed using FlowJo software (Tree Star). Cell sorting of CD4+ T cells was performed on a FACS Aria (BD Biosciences) by labeling non-CD4+ T cells with FITC-conjugated anti-CD4, anti-CD16, anti-CD19, and anti-CD23 (DakoCytomation). Cytokine concentrations in cell supernatants were measured using Ready Set Go ELISA kits (eBioscience).

Statistics

Unless otherwise indicated, data were analyzed using Student’s t test. A value of p < 0.05 was considered statistically significant.

Results

Human CD4+ T cells express TLR3 and TLR9

To examine the expression of TLR3 and TLR9 in the cells used in this study, we isolated human CD4+ T cells from peripheral blood and cultured these for 24 h either in the presence or absence of immobilized anti-CD3 and anti-CD28. We then generated whole cell lysates for analysis by Western blotting and whole cell fractions labeled with anti-TLR3 and anti-TLR9 for analysis by flow cytometry. By Western blotting we found that the CD4+ T cells express both TLR3 and TLR9 and that the expression of both receptors seems to increase in response to activation with immobilized anti-CD3 and anti-CD28 (Fig. 1A). We confirmed these results by flow cytometry where we found an increased number of TLR3- and TLR9-expressing cells in response to stimulation with anti-CD3 and anti-CD28 in comparison to those receiving no stimulation (Fig. 1B). These data show that the activated human CD4+ T cells used in the experiments presented here do express the pattern recognition receptors TLR3 and TLR9, which bind to dsRNA and unmethylated DNA, respectively.
Poly(I:C) induces synthesis of IL-17A and IL-21 in human CD4+ T cells through TLR3

Having established that activated CD4+ T cells express both TLR3 and TLR9, we wanted to investigate whether these receptors could directly induce human CD4+ T cells to produce the cytokines IL-17A and IL-21. To do this, we isolated human CD4+ T cells by depletion of non-CD4+ T cells using magnetically labeled Abs (MACS). Isolated CD4+ T cells were either activated with plate-bound anti-CD3/anti-CD28 or cultured without stimuli (Unstim.) for 24 h. A, Cultured cells were lysed and analyzed using Western blotting. B, Cells were harvested and permeabilized before staining for TLR3 and TLR9 and subsequent analysis by flow cytometry. Gates were determined based on isotype staining. Experiments are representative of independent experiments with cells from three different donors.

FIGURE 1. Human CD4+ T cells express TLR3 and TLR9. Human CD4+ T cells were isolated by depletion of non-CD4+ T cells using magnetically labeled Abs (MACS). Isolated human CD4+ T cells were either activated with plate-bound anti-CD3/anti-CD28 or cultured without stimuli (Unstim.) for 24 h. A, Cultured cells were lysed and analyzed using Western blotting. B, Cells were harvested and permeabilized before staining for TLR3 and TLR9 and subsequent analysis by flow cytometry. Gates were determined based on isotype staining. Experiments are representative of independent experiments with cells from three different donors.

Poly(I:C) induces IL-21 and IL-17A synthesis in activated human CD4+ T cells. A–D and G. Human CD4+ T cells were isolated by depletion of non-CD4+ T cells using magnetically labeled Abs (MACS). Isolated CD4+ T cells were then activated in culture with plate-bound anti-CD3/anti-CD28 and cultured for either 20 or 72 h in the presence or absence (Ctrl) of TLR ligands (poly(I:C), 25 μg/ml; CpG, 2 μM). A and B, mRNA levels of IL-17A and IL-21 were measured using RQ-PCR with B2M as normalizing transcript. The y-axis shows the ratio between primary transcript and B2M. C and D, Secretion of cytokines to the supernatant was measured by ELISA. E and F, CD4+ T cells were either isolated using MACS technology as previously described or by using FITC-labeled Abs and sorting by cell sorter (FACS). Isolated CD4+ T cells were then activated as in A. mRNA levels of IL-17A and IL-21 were measured from both the MACS- and FACS-isolated cells. G, mRNA levels of IL-17A and IL-21 were measured at 20 h from cultures that were preincubated with 5 μM chloroquine for 1 h before the addition of poly(I:C) and from cultures that received no pretreatment with chloroquine. Bars show reduction in mRNA levels caused by chloroquine treatment. Columns and error bars show mean and SEM. Experiments (A–D and G) show data from independent experiments using cells from five to eight different healthy donors. Experiments (E and F) show one experiment and bars show here the range of triplicates. Statistical significance is indicated in the figures.

20 h after TCR stimulation (Fig. 2, A and B). Increases in mRNA were still present at 72 h and for both time points the increases in mRNA were mirrored by increases in protein secretion to the culture supernatants (Fig. 2, C and D). Stimulation with the TLR9 ligand CpG significantly increased IL-21 secretion to the supernatant but did not significantly increase the mRNA levels. CpG did not significantly affect IL-17A on either mRNA or protein levels (Fig. 2, A–D). In a single experiment, we investigated whether the increases in IL-17A and IL-21 in response to poly(I:C) was reproducible when using cells sorted by cell sorter (FACS) instead of
MACS-sorted CD4⁺ T cells. In this study, we found that IL-17A and IL-21 were increased to identical levels in MACS-sorted cells (Fig. 2, E and F). In addition to TLR3, which is located in the endosomes, the cytosolic receptor retinoic acid-induced gene 1 (RIG-I) and melanoma differentiation associated gene 5 (MDA-5) also recognize dsRNA in the form of poly(I:C). The increases in IL-17A and IL-21 production in response to poly(I:C) might therefore be due to these receptors and not TLR3. To clarify this, we examined the ability of the drug chloroquine to inhibit the effects of poly(I:C). Chloroquine interferes with the function of the endosomes and is known to inhibit signaling from endosomal TLRs including TLR3 (46), but not cytosolic receptors such as RIG-I and MDA-5. We found that chloroquine was able to significantly reduce both IL-17A and IL-21 mRNA in CD4⁺ T cells activated in the presence of poly(I:C) (Fig. 2G). From these results, we concluded that poly(I:C) induces human CD4⁺ T cells to synthesize IL-17A and IL-21 through activation of TLR3 and not through activation of cytosolic receptors. Since TLR3 seemed to be the stronger activator compared with TLR9, we chose to focus on TLR3 for the remaining parts of this report.

**Poly(I:C)-induced cytokine synthesis depends on NF-κB and SAPK pathways**

TLR3 operates through at least three major signal transduction pathways. Two of these depend on a signaling complex consisting of TNFR-associated kinase (TRAF) 6, IL-1R-associated kinase 1, and IL-1R-associated kinase 4 and leads to activation of the NF-κB complex with successive nuclear translocation of NF-κB subunits (p65) and to the activation of the stress-activated kinases (SAPK) JNK, and p38 which govern the nuclear accumulation of the AP-1 subunit c-Jun (5, 6). The third pathway depends on a signaling complex consisting of TRAF3, TBK1, and IKKε and leads to activation and nuclear translocation of IRF3 (7). To investigate whether one or more of these pathways could be responsible for the induction of IL-17A and IL-21 synthesis by poly(I:C), we isolated CD4⁺ T cells and activated these with anti-CD3 and anti-CD28 for 16 h. Cells were then washed and rested in medium without stimulation for an additional 8 h. Rested cells were then stimulated with poly(I:C) for 0, 30, 60, 90, and 120 min. Nuclear extracts were prepared and subjected to analysis for IRF3, c-Jun, and p65 by Western blotting. In this study, we found that IRF3, c-Jun, and p65 levels were increased in the nuclear extracts in response to poly(I:C) treatment (Fig. 3A). These results appointed all three transcription factors as potential regulators of IL-17A and IL-21 synthesis. To investigate this in further detail, we measured the effects of specific inhibitors of NF-κB (NBD), p38 (SB203580), and JNK (SP600125) on IL-17A and IL-21 synthesis. We activated isolated CD4⁺ T cells with immobilized anti-CD3 and anti-CD28 for 2 h. Inhibitors were then added to the cultures and incubated for an additional 60 min before adding poly(I:C). IL-17A and IL-21 mRNA levels were measured by RQ-PCR after 20 h of total incubation. We found that NBD significantly decreased IL-17A and IL-21 mRNA levels with >10-fold in both cases. SP600125 and SB203580 significantly affected IL-21 mRNA levels. Addition of SP600126 decreased IL-21 mRNA levels with >10-fold, whereas the addition of SB203580 increased IL-21 mRNA levels by ~3-fold (Fig. 3B). From these results, we concluded that the NF-κB, SAPK, and IRF3 pathways are activated in response to poly(I:C) treatment of human CD4⁺ T cells. Moreover, these results show that the induction of IL-17A and IL-21 both depend on NF-κB and that p38 and JNK regulate the synthesis of IL-21. Due to the absence of commercially available specific inhibitors of IRF3 activation, we were unable to verify the role of IRF3 in the induction of IL-17A and IL-21.

**Poly(I:C) induces differentiation of naive CD4⁺ T cells into an IL-21-producing phenotype**

We next evaluated whether the TLR3-induced increases in IL-21 and IL-17A synthesis were due to a de novo differentiation of naive CD4⁺ T cells into IL-17A- and IL-21-producing subsets. We therefore isolated naive (CD45RA⁺) CD4⁺ T cells using MACS and stimulated these in the presence or absence of poly(I:C).
We then measured the cytokine-producing potential of the cultured cells through activation with PMA/ionomycin. By multiparameter flow cytometry, we found that poly(I:C) increased the number of naive CD4⁺ T cells with the potential to produce IL-21 (Fig. 4A). In contrast, we did not see an increase in the number of naive CD4⁺ T cells with the potential to produce IL-17A in response to poly(I:C) (Fig. 4A). To identify whether the increased number of cells with the potential to produce IL-21 could be accounted for by a differentiation into one of the three currently defined CD4⁺ T cell subsets (T₅₁, T₅₂, and T₅₁十七), we cultured the naive CD4⁺ T cells in the presence or absence of TLR ligands. We then measured mRNA levels of IL-21, IL-17A, T-bet (T₅₁), GATA-3 (T₅₂), and RORC (T₅₁十七). In parallel with what was shown by flow cytometry, the naive CD4⁺ T cells significantly increased mRNA levels of IL-21 in response to poly(I:C) while IL-17A mRNA levels were below detection levels in the cultures that received poly(I:C) as well as in those that did not (Fig. 4B). T-bet and GATA-3 mRNAs were clearly induced in the naive CD4⁺ T cells by stimulus with immobilized anti-CD3 and anti-CD28 but their mRNA levels remained unchanged in response to poly(I:C) treatment (Fig. 4C). In agreement with the absence of detectable IL-17A synthesis, the RORC mRNA levels were also undetectable in cells from both cultures (Fig. 4C). From these experiments, we concluded that poly(I:C) induced a differentiation of naive CD4⁺ T cells into an IL-21 but not into an IL-17A-producing subset. This differentiation was not accompanied by increases in mRNA levels of factors normally associated with the currently defined CD4⁺ T cell subsets T₅₁, T₅₂, and T₅₁十七.

**Poly(I:C) induces expression of IL-17A in memory CD4⁺ T cells**

In the previous sections, we showed that poly(I:C) increased the synthesis of both IL-17A and IL-21 in mixed cell cultures with both naive and memory CD4⁺ T cells (Fig. 2, A–D) but that IL-17A synthesis was absent from the naive CD4⁺ T cells (Fig. 4, A and B). We therefore wanted to investigate the effects of poly(I:C) on memory CD4⁺ T cells. To do this, we isolated memory CD4⁺ T cells (CD45RO⁺) using MACS and cultured these for 20 h in the presence or absence of polo(I:C). We then measured transcription as well as secretion of IL-17A and IL-21. We found that the memory CD4⁺ T cells increased the synthesis of IL-17A in response to poly(I:C) as measured by both RQ-PCR and ELISA (Fig. 5, A and B). The memory CD4⁺ T cells clearly produced IL-21 on both mRNA and protein levels. This synthesis, however, seemed to be insensitive to stimulation with poly(I:C). We then examined whether poly(I:C) treatment induced a skewing of the CD4⁺ T cells toward a T₅₁十七 profile. To do this, we measured the mRNA levels of the transcription factors T-bet (T₅₁), GATA-3 (T₅₂), and RORC (T₅₁十七) by RQ-PCR and measured whether poly(I:C) treatment was able to alter the potential to produce IL-17A in response to PMA and ionomycin by flow cytometry. In this study, we found, that the increase in IL-17A synthesis was not paralleled by an altered pattern in mRNA levels of the transcription factors RORC, T-bet, and GATA-3 (Fig. 5C). Moreover, poly(I:C) treatment did not result in an increased number of either IL-17A- or IL-21-producing memory CD4⁺ T cells (Fig. 5D). From these data, we...
concluded that memory CD4+ T cells respond to poly(I:C) with increased synthesis of IL-17A but not with the alteration of cytokine-producing potential and that although IL-21 is produced by memory T cells its synthesis is here insensitive to poly(I:C). Poly(I:C) induces the development of an IL-21posCXCR3posCCR4neg phenotype

Having established that TLR3 can regulate the synthesis of IL-17A and IL-21, we wanted to explore the surface phenotype of these subsets with focus on the expression of receptors that are associated with autoimmunity. To achieve this, we cultured naive and memory CD4+ T cells separately in either the presence or absence of poly(I:C) for 72 h. By multiparameter flow cytometry, we then analyzed the cultured cells for the expression of either IL-17A or IL-21 combined with surface staining for the chemokine receptors CXCR3 and CCR4. CXCR3 is essential for the migration of T cells into inflamed joints, whereas CCR4 is associated with the migration of T cells into inflamed skin (47, 48). In cell cultures of naive CD4+ T cells, we found that IL-21 and CXCR3 were coexpressed on 71% of the IL-21-producing cells and that this subset of IL-21posCXCR3pos cells increased in response to poly(I:C) (Fig. 6A). In contrast to the coexpression of CXCR3 and IL-21, CCR4 and IL-21 were expressed primarily by separate cell subsets (Fig. 6A). Within the memory CD4+ T cells, we found that the IL-17A-producing cells all coexpressed CCR4 but not CXCR3 (Fig. 6B). In this study, the distribution of CXCR3 and CCR4 expression in the IL-21-producing subset was pleiotropic but comprised both IL-21posCCR4neg and IL-21posCXCR3pos populations (data not shown). In the memory CD4+ T cells, the expression of both CXCR3 and CCR4 were largely unaffected by poly(I:C) treatment. From these chemokine receptor studies, we concluded that naive CD4+ T cells which differentiate in response to poly(I:C) treatment uniformly express CXCR3 and not CCR4. Moreover, we show that this phenotype is not restricted to our in vitro cultures but also exists in the in vivo-differentiated memory CD4+ T cell pool.

Discussion

The induction of inflammation in autoimmune diseases through the ligation of TLRs with microbial ligands fits well with the longtime suspicion of virus either initiating or facilitating the development of autoimmunity (49, 50). Moreover, the ability of TLRs to also recognize endogenous ligands from necrotic cells provides a conceivable explanation for the prolonged and chronic inflammation which causes disabilitating tissue destruction in these diseases (51, 52). These scenarios are supported by the discovery that TLR ligands are indeed present at sites of inflammation in patients with autoimmune diseases and by the increased expression of TLRs in the adjacent tissues (8, 11, 53). In RA, this hypothesis is supported by a recent study in which mRNA from necrotic material from inflamed joints of RA patients was sufficient to induce the production of inflammatory cytokines by synovial fibroblasts via TLR3 (8). However, although macrophages and fibroblasts are likely to respond to
TLR ligands in autoimmunity, other cells might share this feature. During disease, CD4⁺ T cells infiltrate the synovium of RA (13), the skin in psoriasis (54), and the lungs in pulmonary fibrosis and atopic asthma (55, 56). CD4⁺ T cells are therefore likely to encounter TLR ligands that are present at sites of inflammation in these diseases. The potential effects of such encounters are poorly investigated, especially with regard to the production of cytokines that are related to autoimmunity.

**FIGURE 6.** Poly(I:C) induces an IL-21⁺CXCR3⁺CCR4⁻ phenotype in naive Th cells. Isolated naive (A) and memory (B) CD4⁺ T cells were isolated as described previously and cultured separately. Cells were activated in culture with plate-bound anti-CD3/anti-CD28 in the presence or absence (Ctrl) of 25 μg/ml poly(I:C). After 72 h of culture, cells were harvested, washed, and reactivated with 30 ng/ml PMA and 1 mg/ml ionomycin for 6 h. Of these, the last four were in the presence of brefeldin A. Cells were stained with surface markers (CXCR3 and CCR4) before permeabilization and staining for IL-17A and IL-21. Cells were subsequently analyzed by flow cytometry. Diagrams are representative of independent experiments using cells from three different healthy donors.
In the present study, we found that the TLR3 ligand poly(I:C) induced the synthesis of IL-17A and IL-21 from human CD4+ T cells. We also found that poly(I:C) induced synthesis of IL-17A and IL-21 depended on activation of the NF-κB pathway. Together with the increased accumulation of p65 in response to poly(I:C) treatment, our data suggest that poly(I:C) induces IL-17A and IL-21 synthesis by sustaining or increasing nuclear accumulation of p65 through activation of the NF-κB pathway. The ability of poly(I:C) to activate this pathway has been reported elsewhere. In this study, poly(I:C) increased survival of CD4+ T cells by mechanisms that also depended on the NF-κB pathway (16). The SAPKs p38 and JNK regulate the activation of c-Jun which we showed to accumulate in the nucleus in response to poly(I:C). The antagonizing effects of p38 and JNK on IL-21 synthesis could therefore be mediated through regulation of c-Jun activation. The involvement of p38 and JNK in inflammatory disorders has been documented before (57) and our data that they also regulate IL-21 synthesis support the hypothesis that IL-21 plays an important role in autoimmunity. In addition to activation by poly(I:C), cJun and p65 also accumulate in the nucleus in response to activation through a variety of other receptors including the TCR and several of the pathogen recognition receptors that recognize pathogen-associated microbial patterns. It is therefore unlikely that nuclear accumulation of these factors in response to poly(I:C) can deliver a signal that is distinct for differentiation into the IL-21 subset. IRF3, which also accumulated in the nucleus in response to poly(I:C), is not activated by pathways downstream of TCR but relies on signaling from a selection of pathogen recognition receptors. IRF3 or downstream signaling molecules could therefore provide the specificity that induces differentiation into this particular subset. The hypothesis that IRFs direct T cell differentiation is supported by the finding that IRF4, which is up-regulated in response to TLR stimulation (58), is necessary for differentiation into the Th17 subset (59).

In vivo release of IL-17A through TLR3-activated pathways at sites of injury/infection is likely to increase inflammation and to elevate disease activity through activation of endothelia cells and recruitment of neutrophils. Release of IL-21 could activate infiltrating NK cells and CTLs and increase the production of autoantibodies from autoreactive B cells. Our data show that direct activation through TLR3 can induce the release of these cytokines from CD4+ T cells. We therefore suggest that in autoimmune disorders TLRs are not confined to regulate fibroblasts and macrophages but also have the potential to regulate synthesis of proinflammatory cytokines from CD4+ T cells.

Chloroquine was first appreciated in the treatment of malaria but is now a common drug in the treatment of autoimmune diseases. Its inhibition of poly(I:C)-induced synthesis of IL-17A and IL-21 shown in this study not only shows that the effect of poly(I:C) was mediated through TLR3 and not cytosolic helicases, but might also add to understanding of how chloroquine asserts its effects in the treatment of these diseases. If the activation through TLR3 is a significant source of IL-17A and IL-21 in vivo, one of the beneficial effects of chloroquine could be to decrease the production of these cytokines by decreasing the efficiency of the TLR3 pathway.

The TLR9-mediated effects on IL-17A and IL-21 as documented in our report were minor and did not result in a vivid development of a novel phenotype. An involvement of TLR9 in autoimmune diseases is however strongly argued for by its association with, for example, SLE and multiple sclerosis, but more work is needed to assess whether the direct effects of TLR9 on CD4+ T cells are relevant in these diseases.

Importantly, our work showed that TLR3 activation induced the development of an IL-21-producing phenotype from naive CD4+ T cells. The absence of increased mRNA levels of the Th1, Th2, and Th17 hallmark transcription factors T-bet, GATA-3, and RORC suggests that this IL-21 phenotype induced by poly(I:C) does not belong to either of these subsets. These results agree with the recent work of Suto et al. (38), which implied that IL-21- and IL-17(A + F)-producing cells belong to discrete CD4+ T cell subsets. The inability of poly(I:C) to induce increased synthesis of IL-21 in the subset of IL-21-producing memory CD4+ T cells could be due to a number of reasons. The memory cells could have an altered responsiveness to poly(I:C) or altered function of the TLR3 pathway. A similar scenario exists with the need for co-stimulatory receptors. In this study, naive CD4+ T cells responded vividly, whereas memory CD4+ T cells seemed less sensitive to costimulation (60, 61). The expression of CXCR3 on the TLR3-differentiated IL-21-producing CD4+ T cells suggests that TLR3 is important in autoimmune diseases which depend on CXCR3 ligands for recruitment of CD4+ T cells to sites of inflammation (8, 13). This is supported by studies showing that activation of TLR3 leads to the production of CXCR3 ligands CXCL9, CXCL10, and CXCL11 from endothelia and fibroblasts (62, 63). The absence of skin-homing receptor CCR4 on the naive CD4+ T cells suggests that activation through TLR3 alone does not primarily promote the migration of CD4+ T cells to the skin and proposes that CD4+ T cells that are differentiated by activation through TLR3 might be less involved with autoimmune diseases of the skin. The heterogeneity we found in chemokine expression of the memory CD4+ T cells was expected since these cells were differentiated from naive CD4+ T cells in vivo under unknown and heterogeneous conditions, resulting in the acquisition of differential tissue-homing properties. However, the expression of CCR4 on IL-17A-producing memory CD4+ T cells is in agreement with what has been described elsewhere (18) and fits well with the association of IL-17A-producing CD4+ T cells with diseases of the skin and with arthritis (13, 63–66).

Our results identify a novel route for induction of IL-17A and IL-21 in human CD4+ T cells. This route was mediated by direct activation of TLR3 and depended on NF-κB activation and on regulation by SAPKs p38 and JNK. In addition, we have shown that this pathway induces a differentiation of naive CD4+ T cells into an IL-21-producing phenotype which coexpresses surface CXCR3 and CXCL11. This differentiation is supported by studies showing that activation through TLR3 alone does not primarily promote the migration of CD4+ T cells to the skin and proposes that CD4+ T cells that are differentiated by activation through TLR3 might be less involved with autoimmune diseases of the skin.

Disclosures

The authors have no financial conflict of interest.

References


