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A systemic defect in Toll-like receptor 4 signaling increases lipopolysaccharide-induced suppression of IL-2-dependent T-cell proliferation in COPD

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Knobloch J, Chikosi SJ, Yanik S, Rupp J, Jungck D, Koch A. A systemic defect in Toll-like receptor 4 signaling increases lipopolysaccharide-induced suppression of IL-2-dependent T-cell proliferation in COPD. Am J Physiol Lung Cell Mol Physiol 310: L24–L39, 2016. First published October 23, 2105; doi:10.1152/ajplung.00367.2014.—The susceptibility to bacterial infections is increased in chronic obstructive pulmonary disease (COPD). This promotes exacerbations. IL-2 triggers CD4+Th1-cell proliferation, which is important for infection defense. Bacterial endotoxin (LPS) activates MyD88/IRAK and TRIF/IKKε/TBK1 pathways via Toll-like receptor-4 (TLR4) in Th1 cells. Systemic defects in TLR pathways in CD4+Th1 cells cause an impairment of IL-2-dependent immune responses to bacterial infections in COPD. Peripheral blood CD4+ T cells of never smokers, smokers without COPD, and smokers with COPD (each n = 10) were ex vivo activated towards Th1 and stimulated with LPS. IL-2, MyD88, and TRIF expression, and cell proliferation was analyzed by ELISA, quantitative RT-PCR, and bromodeoxyuridine (BrdU) and trypan blue staining comparative among the cohorts. IL-2 release from activated T cells was increased in COPD vs. smokers and never smokers. LPS reduced IL-2 expression and T-cell proliferation. These effects were increased in COPD vs. never smokers and inversely correlated with FEV1 (%predicted). The MyD88/TRIF ratio was decreased in Th1 cells of COPD. The suppression of IL-2 by LPS was abolished by MyD88/IRAK blockade in never smokers but by TRIF/IKKe/TBK1 blockade in COPD. Moxifloxacin restored IL-2 expression and T-cell proliferation in the presence of LPS by blocking p38 MAPK. The increased IL-2 release from Th1 cells in COPD might contribute to airway inflammation in disease exacerbations. A switch from MyD88/IRAK to TRIF/IKKe/TBK1 signaling amplifies the suppression of IL-2-dependent proliferation of CD4+ T cells by LPS in COPD. This molecular pathology is of systemic origin, might impair adaptive immune responses, and could explain the increased susceptibility to bacterial infections in COPD. Targeting TLR4-downstream signaling, for example, with moxifloxacin, might reduce exacerbation rates.

CD4+/Th1 lymphocytes; lipopolysaccharide; COPD exacerbation; Toll-like receptor signaling

CHRONIC OBSTRUCTIVE PULMONARY DISEASE (COPD) is characterized by reversible airflow limitation due to chronic airway inflammation and systemic inflammation that is mostly caused by cigarette smoking. Acute bacterial infections, e.g., with nontypeable Haemophilus influenzae (NTHi), can induce exacerbations, which trigger disease progression and increases in airway and systemic inflammation. Current therapies can at best hinder disease progression (46, 52). Treatment guidelines recommend the use of antibiotics, like the fluoroquinolone moxifloxacin, to treat bacterial infections in COPD (40). Fluoroquinolones have immunomodulatory properties that are thought to influence infection defense mechanisms in COPD (9). Immune responses to bacterial infections depend on the activation of immune cells by pathogen-associated molecular patterns (PAMPs) like lipopolysaccharide (LPS) of gram-negative bacteria. COPD subjects have an increased susceptibility to bacterial infections that suggests defects in their infection defense mechanisms (3, 41). Unraveling these defects would likely provide a basis for developing alternative therapeutic strategies that would help to reduce exacerbation frequencies.

LPS is recognized by the transmembrane Toll-like receptor-4 (TLR4) that is expressed on immune cells. Activated TLR4 signals via two pathways to regulate the expression of cytokines: the MyD88/IRAK and the TRIF/IKKe/TBK1 pathway. Activated TLR4 forms homodimers, which allow MyD88 and TRIF in a complex with TIRAP or TRAM, respectively, to bind to the cytosolic TIR domains of the receptor dimer. MyD88 and TRIF are the key adaptor molecules of this signaling network because they determine which downstream pathway is used. They do not have enzymatic activities alone but after binding they recruit downstream protein kinases, IRAK-4 and IRAK-1 or IKKe and TBK-1, respectively. These protein kinases further transduce the signal via various signaling molecules to finally activate transcription factors like NF-κB. The MyD88 pathway mediates early and the TRIF pathway late NF-κB activation. Cross talk between the two pathways is possible (8, 42).

We believe that the accumulation of smoking-dependent and -independent local defects in innate immune responses and of systemic defects in adaptive immune responses impairs the defense against acute bacterial infections in the lung of COPD subjects. There is plenty of evidence for the dysregulation of innate immune cells in COPD (13). However, data on systemic defects in adaptive immune responses are limited. This raises the question of a suitable experimental model to address this
issue. In the absence of acute infections, ~95% of the circulating CD4^+ T cells in COPD and healthy subjects are non-effectector naive or memory T cells (36, 37). In response to acute bacterial airway infections, antigen-presenting cells enter the peripheral draining lymph nodes near to the site of infection. There they stimulate circulating non-effectector CD4^+ T cells that recognize the antigen to produce IL-2, to proliferate, and to differentiate towards Th1 (T-helper cell type 1). T-cell proliferation depends on IL-2. The resulting Th1 effector cells trigger the adaptive immune response to bacterial pathogens for which IFNγ and IL-2 are crucial. To do this, they either stay in the draining lymph nodes or enter the infected airway tissue (15, 18, 31, 35, 51, 53). Th1 cells generated ex vivo from circulating CD4^+ T cells are a suitable cell culture model to elucidate systemic T-cell defects in COPD. It reflects the activation/differentiation of circulating CD4^+ T cells into Th1 cells in vivo that takes place in response to acute infections.

The effector activities of ex vivo-generated Th1 cells resemble the effector activities of those Th1 cells in vivo, which become recruited into the airways in response to an acute airway infection. Adding LPS to the culture mimics the exposition of CD4^+ T cells to bacteria and bacterial fragments during proliferation and differentiation. In vivo this exposition takes place not only in the infected tissue but also in the draining lymph nodes. Therefore, bacteria can influence the activity of T cells in vivo at any stage of development. In the culture model, LPS is added a short time after T-cell activation. Therefore, it reflects primarily the influence of bacteria on early T-development including proliferation. We have shown before that Th1 cells express functional TLR4 and can directly respond to LPS (31). Therefore, this ex vivo culture model is suitable for investigating systemic T-cell defects with relevance for local infection defense (31). In contrast, the persistent effector activities of resident T cells that can be found in excess in the lung of COPD subjects in the absence of an acute infection are not directed against new infections (6, 14, 21). Consequently, investigating airway Th1 cells might have less relevance for defense mechanisms against acute infections.

Using this ex vivo culture model, we have shown that a smoking-dependent and systemic overexpression of endogenous antagonists of the TLR4/MD2/IRAK pathway in circulating CD4^+ T cells prevents TLR4 and IFNγ upregulation in direct response to LPS and NTHI after activation towards Th1 in COPD. This suggests that a systemic molecular pathology in circulating CD4^+ T cells causes an impaired Th1 immune response to acute respiratory tract bacterial infections in COPD (7, 31). From this, we assume that molecular defects in TLR signaling in circulating T cells, which impair their response to LPS, contribute to the reduced infection defense in COPD. These systemic defects become critical when the circulating T cells are recruited into the lung in response to acute infections and come into contact with bacteria and LPS. As a consequence of the impaired infection defense, bacterial clearance is hindered, colonization might occur, and defense mechanisms become hyperactivated repeatedly leading to an increase in airway inflammation and to disease exacerbation.

Compared with the Th1-specific effector cytokine IFNγ, IL-2 influences multiple mechanisms in infection defense: IL-2 triggers T-cell proliferation and differentiation and is required for Th1- and cytotoxic T-cell-dependent but also for Th2-related immune responses. Thus IL-2 is crucial for the defenses against extra- and intracellular bacteria. Th1 cells are the major IL-2 source (15, 35, 53).

We hypothesized that the IL-2-dependent Th1-cell immune response to gram-negative bacterial infection is systemically impaired in COPD. We compared IL-2 release from ex vivo-generated Th1 cells in the absence and presence of LPS among never smokers, current smokers without airflow limitation, and current smokers with COPD. We investigated the molecular pathology underlying the differences between cohorts and the functional consequences on T-cell proliferation, and we aimed at pharmacological restoration of dysregulated IL-2 production and T-cell proliferation in COPD by utilizing the immunomodulatory properties of moxifloxacin.

### MATERIALS AND METHODS

**Study subjects.** The study population consisted of 10 healthy nonsmokers with no smoking history (NS); 10 current smokers (≥10 pack-years) without respiratory symptoms or airflow limitation (S); and 10 current smokers with respiratory symptoms and airflow limitation [COPD; ≥10 pack years, Global Initiative for Chronic Obstructive Lung Disease (GOLD) stages II-IV; NS, nonsmoker; S, current smoker without COPD (≥10 pack years); FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity. *P* < 0.001 vs. S and NS.

<table>
<thead>
<tr>
<th>Variable</th>
<th>NS</th>
<th>S</th>
<th>COPD</th>
<th><em>P</em></th>
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<tbody>
<tr>
<td>Age, yr</td>
<td>55 ± 1.8</td>
<td>53.4 ± 2.9</td>
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<td>Gender (male:female)</td>
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<tr>
<td>FEV₁, %predicted</td>
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<td>97.3 ± 6.0</td>
<td>49.1 ± 4.0*</td>
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<tr>
<td>FEV₁/FVC, %</td>
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<td>79.5 ± 1.8</td>
<td>60.6 ± 2.0*</td>
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<tr>
<td>FVC, %predicted</td>
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<tr>
<td>Pack years</td>
<td>44.5 ± 11.6</td>
<td>46.8 ± 10.5</td>
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Values are means ± SE. Chronic obstructive pulmonary disease (COPD) subjects were Global Initiative for Chronic Obstructive Lung Disease (GOLD) stages II-IV. NS, nonsmoker; S, current smoker without COPD (≥10 pack years); FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity. *P* < 0.001 vs. S and NS.

**Sample size calculation.** Sample size calculation was done with the Russ Lenth’s program for power and sample size calculation. Sample size calculation was based on the following primary outcome: differences in the effect of LPS on IL-2-release from ex vivo-generated Th1 cells among NS, S, and COPD (GOLD stages II-IV). On the basis of preliminary experiments with *n* = 4 subjects of each group (we used relative values; see Fig. 2C for calculation), it was estimated that the sample size to achieve a power of *1 - β* = 0.9 for a one-way ANOVA test at *α* = 0.05 would be nine subjects in each group. We increased the numbers to 10 subjects per cohort.

**Table 1. Demographics of T-cell donors**

<table>
<thead>
<tr>
<th>Groups</th>
<th>NS</th>
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<td>Age, yr</td>
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Values are means ± SE. Chronic obstructive pulmonary disease (COPD) subjects were Global Initiative for Chronic Obstructive Lung Disease (GOLD) stages II-IV. NS, nonsmoker; S, current smoker without COPD (≥10 pack years); FEV₁, forced expiratory volume in 1 s; FVC, forced vital capacity. *P* < 0.001 vs. S and NS.
Isolation and cultivation of peripheral blood CD4+ T lymphocytes. CD4+ T lymphocytes were isolated via Ficoll gradient sedimentation and subsequent two rounds of magnetic bead-based negative selection from the peripheral blood of donors and were cultivated as described previously before (31). Briefly, peripheral venous blood was drawn into sterile syringes (Becton Dickinson, Franklin Lakes, NY) by venipuncture with anticoagulant-citrate-dextrose (ACD) solution as anticoagulant at a final concentration of 10% (vol/vol). The ACD blood was prepared by Ficoll gradient sedimentation as previously described (31, 32). Nonadherent cells (including lymphocytes) were separated from adherent cells by plastic adherence seeding in a culture flask in culture medium in a humidified atmosphere with 5% CO2 for 3 h at 37°C. From the nonadherent fraction, untouched CD4+ T cells were purified with the magnetic bead-based Dynal CD4 Negative Isolation Kit (cat no. 113.46D; Life Technologies, Darmstadt, Germany). The antibody mix used for the depletion of non-CD4+ cells contained mouse IgG antibodies for CD8, CD14, CD16a/b, CD19, CD36, CD56, CDw123, and CD235a. Two rounds of purification were performed. As confirmed by FACS analysis (32), the purity of CD4+ lymphocytes was >98%. Cells were >85% viable as assessed by trypan blue exclusion (31, 32). If not otherwise noted, CD4+ T cells were cultured in RPMI1640 medium (Sigma, Munich, Germany) supplemented with 10% (vol/vol) fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Sigma) as described before (32).

Preparation of NTHi whole cell extracts. The NTHi strain used in this study was a clinical isolate from the University Hospital in Wiesbaden, Germany. LPS (from Salmonella enteritidis; cat. no. 219-IL-005; R&D Systems, Wiesbaden, Germany) and soluble mouse anti-human CD28 monoclonal antibody (cat. no. MAB202, MAB002; R&D Systems) were added 5 min (for p38 MAPK activity assay) or 30 min (for all other analyses) before addition of IL-12 (10 ng/ml; cat. no. 219-IL-005) and supplements as described above for 16 or 48 h without changing the medium. Ex vivo T lymphocytes. T lymphocytes were isolated via Ficoll gradient sedimentation and subsequent two rounds of magnetic bead-based negative selection from the peripheral blood of donors and were cultivated as described previously (31, 32). Nonadherent cells (including lymphocytes) were separated from adherent cells by plastic adherence seeding in a culture flask in culture medium in a humidified atmosphere with 5% CO2 for 3 h at 37°C. From the nonadherent fraction, untouched CD4+ T cells were purified with the magnetic bead-based Dynal CD4 Negative Isolation Kit (cat no. 113.46D; Life Technologies, Darmstadt, Germany). The antibody mix used for the depletion of non-CD4+ cells contained mouse IgG antibodies for CD8, CD14, CD16a/b, CD19, CD36, CD56, CDw123, and CD235a. Two rounds of purification were performed. As confirmed by FACS analysis (32), the purity of CD4+ lymphocytes was >98%. Cells were >85% viable as assessed by trypan blue exclusion (31, 32). If not otherwise noted, CD4+ T cells were cultured in RPMI1640 medium (Sigma, Munich, Germany) supplemented with 10% (vol/vol) fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Sigma) as described before (32).

Stimulation of peripheral blood CD4+ T lymphocytes. Ex vivo generation of Th1 lymphocytes was achieved as described previously (31, 32). Briefly, freshly isolated peripheral blood CD4+ T lymphocytes were stimulated with precoated mouse anti-human CD3 monoclonal antibody (cat. no. 555336; BD Becton Dickinson, Heidelberg, Germany) and soluble mouse anti-human CD28 monoclonal antibody (cat. no. 555725; BD Biosciences) both at 500 ng/ml for 5 min (for p38 MAPK activity assays) or 30 min (for all other analyses) before addition of IL-12 (10 ng/ml; cat. no. 219-IL-005) and R&D Systems, Wiesbaden, Germany). LPS (from Salmonella enteritidis; the preparation contained <1% protein and 10–20% nucleic acids; cat. no. L7770; Sigma) and NTHi whole cell extract were added 5 min (for p38 MAPK activity assays) or 30 min (for all other analyses) after stimulation with IL-12 to the culture medium. Moxifloxacin (Bayex Vital, Leverkusen, Germany), BX795 (10 nM; cat. no. tli-bx7; Invivogen, San Diego, CA), CLI-095 (TAK-242; 1 μM; cat. no. tli-cll95; Invivogen), the IRAK-1/4 inhibitor N-2-morpholinyl-ethyl-2-(3-nitrobenzoylamido)-benzimidazole (10 μM; cat. no. 407601; Calbiochem/VWR, Darmstadt, Germany), the MyD88 inhibitory peptide (RQIKIFQWNRMKWKKRDVLPGTCVSNH2; 25 μM; cat. no. tli-pimyd; Invivogen), the TLR9-specific inhibitory nucleotide ODN TTAGGG (2.5 μM; cat. no. hinohodin; Invivogen; 5ʾ-ttt agg gtt agg gtt agg gtt agg g-3ʾ), Polymyxin B (cat. no. tli-pmb; Invivogen), SB203580 (1 μM; cat. no. 559389; Calbiochem/VWR), the TLR2 neutralizing antibody (10 μg/ml; cat. no. phb-htl2; Invivogen), the TRF inhibitory peptide (RQIKIFQWNRMKWKKFCCEFQVPGRHELH2; 25 μM; cat. no. tli-pitrif; Invivogen), or the IL-2 neutralizing antibody and IgG1 isotype controls (clones 5334 and 11711; cat. no. MAB202, MAB002; R&D Systems) were added 15 min, 30 min, 60 min, or 2 h before stimulation with anti-CD3/anti-CD28 antibodies or LPS to the cells. In some experiments the FCS in the culture medium was replaced by 10% human peripheral blood serum pooled of n = 4 NS, n = 4 S, or n = 4 COPD (Table 2). Whole blood was collected into Vacutainer tubes. The serum was prepared by centrifugation at 1,500 g for 10 min. Serum of n = 4 subjects of a cohort was pooled to equal shares and stored at −80°C until it was used for preparing the culture medium. Then, 5 × 107 T cells of NS, S, or COPD were preincubated in 250 μl medium with 10% pooled human serum of NS, S or COPD and supplements as described above for 48 h in 1.5-ml reaction tubes (with open lids) in a humidified atmosphere with 5% CO2 at 37°C before they were seeded into culture plates that were precoated with mouse anti-human CD3 monoclonal antibodies. T-cell activation was done as described above for 16 or 48 h without changing the medium. Transfection experiments. The TLR4 expression construct (wild-type TLR4), the control vector (pCX-GFP), and the protocol for the transfection of DNA vectors into CD4+ T cells by electroporation is described in Ref. 31. Nontransfected CD4+ T cells and CD4+ T cells transfected with wild-type TLR4 or with the control vector were left untreated, stimulated with αCD3/αCD28/IL-12, or stimulated with αCD3/αCD28/IL-12 plus LPS as described above.

RNA isolation and quantitative RT-PCR. DNA-free total RNA was extracted from CD4+ T cells with the chromatography-based RNeasy technique (Qiagen, Hilden, Germany). Quantitative RT-PCR (qRT-PCR) was done as described previously (30, 31). Briefly, after cDNA synthesis with random primers, quantitative PCRs were done with gene-specific and (if possible) intron-spanning primers. The housekeeping gene EF1α was used as a reference. PCR conditions were established for each primer pair in the exponential range. This allows the quantification of signal intensities after ethidium bromide staining and standard agarose gel electrophoresis by densitometry using the Alpha Innotech (San Leandro, CA) software, version 1.3.0.7. As a prerequisite for a reliable quantification of signal intensities, the Alpha Innotech software recognizes oversaturation (e.g. due to excess loading of the agarose gel) and nonlinear gamma or contrast correction of signals and disables the densitometry tools in those cases. The data for target genes under the different stimulation conditions were normalized to EF1α reference signals to correct for putative differences in RNA/cDNA load. Primer sequences for EF1α have been published previously (32), primer sequences for IL-2, MyD88, TRIF, IRAK1, and IκKε are available upon request.

IL-2 measurements (ELISA). IL-2 concentrations in supernatants of cultivated CD4+ T lymphocytes (1 × 105 cells/ml culture medium) were measured by ELISA according to the instructions of the manufacturer (cat. no. DY202; R&D Systems) and as described before (38).

Table 2. Demographics of serum donors

<table>
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<th>NS</th>
<th>S</th>
<th>COPD</th>
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<tr>
<td>Age, yr</td>
<td>60.8 ± 3.8</td>
<td>60.8 ± 5.6</td>
<td>58.0 ± 2.7</td>
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<td>Gender (male/female)</td>
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<tr>
<td>FEV1, %predicted</td>
<td>99.0 ± 8.0</td>
<td>99.3 ± 10.2</td>
<td>44.7 ± 7.7*</td>
</tr>
<tr>
<td>FEV1/FVC, %</td>
<td>80.6 ± 1.3</td>
<td>79.9 ± 1.5</td>
<td>51.9 ± 3.4*</td>
</tr>
<tr>
<td>FVC, %predicted</td>
<td>100.6 ± 10.7</td>
<td>100.8 ± 11.4</td>
<td>72.4 ± 12.4</td>
</tr>
<tr>
<td>Pack years</td>
<td>—</td>
<td>62.4 ± 29.8</td>
<td>54.8 ± 8.9</td>
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Values are means ± SE. *P < 0.01 vs. NS and S; †P < 0.001 vs. S and NS.
mouse anti-human-total-p38 MAPK antibody was added for 16 h at 4°C. To distinguish between phosphorylated p38 MAPK and the total cellular p38 MAPK content the respective specific antibodies have to be derived from different species. After being washed, a secondary antibody mixture (horseradish peroxidase-conjugated anti-rabbit IgG and AP-conjugated anti-mouse IgG) was added for 2 h at room temperature. After being washed, horseradish peroxidase-fluorogenic substrate was added for 60 min and, afterwards, AP-fluorogenic substrate was added for 5 min. Phospho-p38 MAPK was measured using a fluorescence plate reader with excitation at 540 nm and emission at 600 nm. Total p38 MAPK was measured with excitation at 360 nm and emission at 450 nm. Values for phospho-p38 MAPK were normalized to values for total p38 MAPK.

Cell counts. Cells counts and cell death were analyzed microscopically by trypan blue staining as described before (31, 32) in a Neubauer chamber. For each probe the mean of the counts of four corner squares was calculated.

Bromodeoxyuridine assay. The incorporation of bromodeoxyuridine (BrdU) into the DNA of T cells was measured as described before (39) with modifications. Briefly, 5 × 10^4 CD4^+ T cells in 50 μl RPMI medium with 10% FCS and supplements as described above were seeded into 96-well plates and activated with precoated anti-human CD3 antibody and soluble anti-human CD28 antibody. After 30 min, IL-12 was added. LPS was added 30 min after IL-12. BrdU was added 24 or 48 h after LPS stimulation for the final 24 h of incubation. BrdU was measured with a BrdU detection kit (cat. no. ab126556; Abcam, Cambridge, UK) according to the instructions of the manufacturer. Samples were run in duplicate.

Statistical analysis. Statistical analyses were performed to examine the effects of stimulating agents and specific inhibitors of signal transduction alone or in combination on gene expression and cytokine release from T lymphocytes comparative among NS, S, and COPD. Histogram analyses were used to test for a Gaussian distribution. The results are expressed as means ± SE (Gaussian distribution) or as median with either scatter or 25th to 75th interquartile range and minimum/maximum (nonparametric distribution). Comparisons across different cohorts and stimulations were analyzed by one way ANOVA, one-way repeated measures ANOVA (Gaussian distribution), Kruskal-Wallis, or Friedman test (nonparametric distribution) across different cohorts and stimulations were analyzed by one way ANOVA, one-way repeated measures ANOVA (Gaussian distribution). Comparisons were normalized to values for total p38 MAPK.

RESULTS

IL-2 release is increased in Th1 cells of COPD subjects. Ex vivo activation and differentiation of nonkiller CD4^+ T cells into Th1 cells (Th1 activation) were achieved by a combined T-cell receptor (CD3) and coreceptor (CD28) activation with agonistic anti-CD3 (αCD3) and anti-CD28 (αCD28) antibodies and additional IL-12 stimulation (31). αCD3/αCD28 stimulation induced a time-dependent IL-2 release from peripheral blood CD4^+ T cells of NS. This was enhanced by additional IL-12 stimulation (Fig. 1A). IL-2 release from unstimulated CD4^+ T cells was without differences among NS, S, and smokers with COPD (data not shown). IL-2 release from ex vivo-generated Th1 cells was stronger in COPD compared with NS and S (Fig. 1B) and inversely correlated to FEV₁ (%predicted) (Fig. 1, C and D) but not to pack years (data not shown). IL-2 triggers T-cell proliferation (35). Accordingly, CD4^+ T-cell counts increased after 72 and 96 h of αCD3/αCD28/IL-12 stimulation (data not shown, Fig. 1E). Unexpectedly, this was without differences among the cohorts. DNA synthesis is a prerequisite for cell division and proliferation and can be marked by BrdU incorporation. αCD3/αCD28/IL-12 stimulation induced the incorporation of BrdU into DNA without differences among the cohorts (Fig. 1F). We conclude that the increase of IL-2 expression in Th1 cells of COPD does not have functional consequences on T-cell proliferation.

LPS suppresses IL-2 stronger in T cells of COPD subjects than in T cells of healthy subjects. LPS suppressed IL-2 release from Th1 cells in all cohorts (Fig. 2, A–C). After 24 and 48 h of stimulation, this was without differences among the cohorts (Fig. 2, A and B). After 72 h, LPS strongly reduced IL-2 release from Th1 cells of COPD but not from Th1 cells of NS and S (Fig. 2C). Despite the stronger LPS effect, the absolute amounts of IL-2 that were produced by Th1 cells after LPS stimulation were still higher in the COPD group compared with NS and S (Fig. 2C). Suppression of IL-2 by LPS after 72 h inversely correlated to FEV₁ (%predicted) (Fig. 2D) but not to pack years (data not shown) suggesting that this long-term effect of LPS in COPD is independent from smoking. LPS did not modulate CD4^+ T-cell counts after 72 h (data not shown) but reduced CD4^+ T-cell counts after 96 h (Fig. 2E). This effect was stronger in COPD compared with NS and S (Fig. 2E) and inversely correlated to FEV₁ (%predicted) (Fig. 2F). LPS did not induce cell death in all cohorts (data not shown). LPS did not significantly modulate BrdU incorporation after 48 h but reduced BrdU incorporation after 72 h. This effect was stronger in COPD compared with NS (Fig. 2G). This matches the cell count data because DNA synthesis precedes cell division.

To test whether LPS-induced suppression of IL-2 was causative for the reduced CD4^+ T-cell counts in response to LPS, we performed two experiments with CD4^+ T cells of COPD. CD4^+ T-cell counts after activation with αCD3/αCD28/IL-12 were reduced by blocking IL-2 with a specific antibody (Fig. 3A). This confirms that IL-2 triggers the proliferation of CD4^+ T cells from COPD. Next, we added different concentrations of recombinant IL-2 after αCD3/αCD28/IL-12 and LPS stimulation in time-response postincubation experiments. LPS reduced IL-2 release from Th1 cells of COPD by ~2 ng/ml (Fig. 2, A–C). Accordingly, the addition of recombinant IL-2 at 2 ng/ml, making up for this difference, prevented the reduction of CD4^+ T-cell counts by LPS in a time-dependent manner in COPD (Fig. 3B). Summarized, although absolute IL-2 levels in COPD compared with NS and S remain higher in the presence of LPS (Fig. 2C), the stronger reduction (in percent) of IL-2 by LPS in COPD leads to a stronger reduction of CD4^+ T-cell proliferation.

This suggests that in COPD, despite an excess of persistently activated T cells in the lungs, the number of antigen-specific Th1 cells that are newly recruited into the lung in response to acute bacterial infections is reduced. As this could pose a critical defect in infection defense, we aimed at unraveling the underlying molecular pathology.
Suppression of IL-2 by LPS depends on baseline but not on LPS-induced TLR4 expression. The suppression of IL-2 by LPS was completely abrogated by the LPS-neutralizing reagent Polymyxin B and by the TLR4-specific inhibitor CLI-095 but was not affected by blockade of TLR2 or TLR9 (Fig. 4A). This demonstrates that the effects of LPS on IL-2 were not due to putative contaminating reagents in LPS preparations and that LPS blocks IL-2 expression via TLR4. NTHi total-extract suppressed IL-2 release from Th1 cells, which was reversed by Polymyxin B and CLI-095 (Fig. 4B). Thus gram-negative bacteria suppress IL-2 in Th1 cells primarily via LPS/TLR4 signaling.

TLR4 baseline expression in ex vivo-generated Th1 cells is not influenced by smoking or COPD. LPS increases TLR4 on Th1 cells of NS but not in COPD (31). LPS-induced IFNγ expression in Th1 cells depends on this TLR4 upregulation and, therefore, is completely abolished in COPD (31). The suppression of IL-2 by LPS is stronger in COPD (Fig. 2C). This can only be brought in agreement with the IFNγ data if IL-2 regulation by LPS exclusively depends on baseline but not on LPS-induced TLR4 expression. To test this hypothesis, CD4+ T cells of COPD were transfected with an expression construct encoding human wild-type TLR4 or with a GFP control vector before activation towards Th1. TLR4 overexpression functionally mimics LPS-induced TLR4 expression (31). Independent from the construct and stimulation, transfected cells released slightly smaller cytokine amounts than nontransfected controls because the electroporation technique used for transfection caused cell death in 30% of the cells (31) (Fig. 5). As shown before (31), Th1 cells of COPD with the TLR4 vector but not those with the control vector or nontransfected cells showed a strong IFNγ response to LPS stimulation, demonstrating functionality of overexpressed TLR4 (Fig. 5A). However, TLR4 overexpression did not influence the suppression of IL-2 by LPS (Fig. 5B). This demonstrates that TLR4 baseline expression is sufficient to mediate the full IL-2 response to LPS and that IL-2 regulation is independent from LPS-induced TLR4 expression. Therefore, the data showing a stronger suppression (in percent) of IL-2 by LPS in COPD (Fig. 2C) are not contradicting previous data showing a weaker IFNγ induction by LPS in COPD (31).

LPS activates the MyD88/IRAK pathway in NS but the TRIF/IKKe/TBK1 pathway in COPD to suppress IL-2 transcription. To unravel mechanisms that underlie the stronger suppression (in percent) of IL-2 by LPS in COPD and that...
could be therapeutic targets, we analyzed the expression of TLR4 pathway components comparatively among the cohorts. Activation of canonical MyD88/IRAK or alternative TRIF/IKKe/TBK1 signaling by TLR4 depends on the adaptor protein, MyD88 or TRIF, that is recruited to the receptor (8). MyD88 mRNA baseline levels in CD4+ T cells were without differences among the cohorts (Fig. 6, A and B). TRIF mRNA baseline levels were increased in COPD compared with NS and S (Fig. 6, A and D). Th1 activation did not influence TRIF expression in all cohorts but increased MyD88 expression in NS and S but not in COPD (Fig. 6, A, C, and E). Consequently, Th1 activation increased the MyD88/TRIF mRNA ratio in
CD4\(^+\) T cells of NS and S but not in COPD. This resulted in a decreased MyD88/TRIF ratio in Th1 cells of COPD compared with NS (Fig. 6F). Expression of downstream kinases IRAK1 and IKKe was not modulated by Th1 activation and was not different among the cohorts (data not shown). LPS did not modulate MyD88, TRIF, IRAK1, and IKKe expression in all cohorts (Fig. 6A; data not shown). Summarized, these data indicate a switch from MyD88/IRAK to TRIF/IKK\(\varepsilon\)/TBK1 signaling downstream of TLR4 in Th1 cells of COPD.

To investigate whether dysregulated IL-2 expression in response to LPS in COPD could be explained by this molecular pathology we performed blocking experiments with inhibitors for both pathways and compared the effects between NS and COPD. T cells of S were not tested as the MyD88/TRIF ratio was not different between NS and S. Comparable to the IL-2 protein data (see above), activation of CD4\(^+\) T cells towards Th1 caused an upregulation of IL-2 mRNA, LPS reduced IL-2 mRNA, and these effects were stronger in COPD compared with NS (Fig. 7, A–C). This suggests that LPS suppresses IL-2 transcription and that IL-2 dysregulation in COPD depends on a defective transcriptional regulation. Blocking of the MyD88/IRAK pathway reversed the reduction of IL-2 transcription and protein release by LPS in Th1 cells of NS but not in COPD (Fig. 7, B and D–F). In contrast, inhibition of the TRIF/IKK\(\varepsilon\)/TBK1 pathway had only marginal effects on IL-2 in NS but significantly reversed the reduction of IL-2 transcription and protein release by LPS in Th1 cells of COPD (Fig. 7, B and D–F). This demonstrates that LPS regulates IL-2 transcription primarily via MyD88/IRAK signaling in NS but signals through the TRIF/IKK\(\varepsilon\)/TBK1 pathway in COPD, which might...
activated CD4$^+$ T cells of NS (Fig. 8A). There was no difference in IL-2 production between CD4$^+$ T cells of COPD that were activated in medium with serum of NS, S or COPD (Fig. 8A). We conclude that CD4$^+$ T cells of COPD are saturated regarding the response to the inflammatory proteins in the serum of COPD subjects. Since human serum might contain IL-2, we measured IL-2 in medium controls without T cells but we did not detect any (data not shown). This rules out the possibility that we measured serum IL-2 instead of IL-2 produced by the cultured T cells.

MyD88 but also TRIF expression was without differences between unstimulated CD4$^+$ T cells of NS cultivated in medium with serum of NS, S, or COPD (Fig. 8B, data not shown). However, Th1 cells of NS showed a weaker MyD88 expression when they were cultivated and activated in the presence of serum of COPD subjects than in the presence of serum of NS (Fig. 8B). This shows that in our culture conditions constituents of COPD serum can cause some of the molecular pathologies characteristic for COPD in CD4$^+$ T cells of NS: the IL-2 upregulation and the MyD88 downregulation in Th1 cells. This supports the hypothesis that these molecular pathologies become induced by systemic inflammation in COPD. However, the incubation in serum of COPD subjects did not cause an upregulation of TRIF in unstimulated CD4$^+$ T cells of NS.

Moxifloxacin reverses the suppression of IL-2 and the reduction of T-cell counts by LPS. We also investigated the role of p38 MAPK, another TLR4 downstream kinase. LPS activated p38 MAPK in Th1 cells, and specific p38 MAPK blockade enhanced IL-2 release from LPS-exposed Th1 cells in NS and COPD (Fig. 9, A and B). Thus p38 MAPK suppresses IL-2 expression. LPS-induced p38 MAPK activity was reduced by MyD88- or TRIF-blocking peptides (Fig. 9C). This demonstrates that LPS can activate p38 MAPK via both MyD88 and TRIF signaling to suppress IL-2 expression.

Reduced T-cell counts under conditions with LPS exposure could weaken bacterial infection defense in COPD. Thus we aimed at reversing T-cell counts and IL-2 reduction pharmacologically in our T-cell culture model. We tested moxifloxacin, which is used in COPD exacerbations (40). Moxifloxacin blocked p38 MAPK in Th1 cells significantly in the presence of LPS and by trend in the absence of LPS (Fig. 9B). Moxifloxacin concentration- and time-dependently increased IL-2 release from Th1 cells of NS in the presence and absence of LPS (Fig. 9, D and E). Moxifloxacin induced IL-2 in LPS-exposed Th1 cells in all cohorts thereby neutralizing the suppression of IL-2 by LPS in COPD (Fig. 9F). Moxifloxacin almost completely reversed the reduction of T-cell counts caused by LPS in all cohorts (Fig. 9G) and did not induce cell death (data not shown). This demonstrates that moxifloxacin-induced IL-2 expression can reverse the stronger reduction of T-cell proliferation caused by LPS in COPD. Finally, we compared the effects of moxifloxacin and the p38 MAPK-specific inhibitor SB203580 on IL-2 release in LPS-exposed ex vivo-generated Th1 cells of 23 subjects (8 NS, 8 S, and 7 COPD). By using linear regression, we found a positive correlation of the inducing effects of both drugs on IL-2 ($r^2 = 0.4514$; $P = 0.0004$). This supports the concept that moxifloxacin induces IL-2 expression by blocking p38 MAPK activity.
DISCUSSION

Susceptibility to bacterial infections is increased in COPD. Unraveling the underlying immunologic defects provides a basis for developing alternative therapies for COPD exacerbations. Acute exacerbations can result from acute bacterial airway infections (that may be secondary to viral infections) and from bacterial colonization (46). Here, we investigated our hypothesis that IL-2-dependent adaptive immune responses to acute bacterial infections are impaired in COPD. COPD is a systemic disease and systemic defects in adaptive immune cells might impair local infection defenses (1, 17, 31). We found two immunologic pathologies in COPD.

First, IL-2 production of Th1 cells but not of noneffector CD4+ T cells was increased in COPD in correlation to lung function. This was independent from tobacco consumption. As T-cell activation towards Th1 is a consequence of acute and chronic infection, this might contribute to the amplification of inflammation in infection-caused COPD exacerbations. Considering the failure to clear T cells from the airways after infections (6, 27), IL-2 hyperproduction of Th1 cells might also contribute to chronic inflammation in stable COPD. Our data match with other studies demonstrating a hyperactivation of immune cells including T cells in COPD (5, 14, 45).

IL-2 is the major T-cell growth factor (15, 35, 53). Surprisingly, the increased IL-2 production of Th1 cells of COPD subjects was not associated with an increased Th1 cell proliferation, as determined by counting T cells and measuring DNA synthesis. Suboptimal culture conditions as a technical explanation can be ruled out since neither the addition of recombinant IL-2 nor decreasing the number of cells per unit culture area in our ex vivo-activation experiments resulted in an increase T-cell counts (Fig. 3B, data not shown). Thus the IL-2 amounts that are produced by CD4+ T cells of healthy subjects after activation are sufficient to induce maximal T-cell proliferation. Using IL-2 neutralizing antibodies we have confirmed that the proliferation of T cells of COPD subjects depends on IL-2. We conclude that CD4+ T cells in COPD do not have the capacity to respond to their increased IL-2 production with an increase in proliferation. We hypothesize that the IL-2/IL-2 receptor system is already saturated at lower IL-2 concentrations, more specifically, by the amount of IL-2 that is produced by activated T cells of NS. In support of this,
the addition of recombinant IL-2 at any time to CD4\(^+\) T cells stimulated with αCD3/αCD28 and IL-12 did not increase cell counts (Fig. 3B).

Second, LPS suppressed IL-2 in Th1 cells, which caused a reduction of cell proliferation. Both effects were stronger in COPD, were inversely correlated to lung function, and were independent from tobacco consumption. This provides evidence that bacteria partially block T-cell proliferation and that this immunosuppressive mechanism is augmented in COPD. During airway infections, bacteria and bacterial fragments are present in the draining lymph nodes and particularly under conditions of inflammation effector T cells enter the infected lung tissue (18). Thus T cells can come in contact with bacterial PAMPs at any stage of development after initial activation in response to acute airway infections. This suggests that our cell culture data have relevance in vivo. We have shown previously that the capacity of Th1 cells to produce IFN\(γ\) in direct response to bacteria and to LPS is reduced in COPD but also in active smokers without COPD compared with healthy never smokers (31). Together with the data from this study and despite the excess of resident lung T cells, this suggests a lack of antigen-specific Th1 cells that are newly recruited into the lung and of IFN\(γ\) production by these cells in response to acute bacterial airway infections in COPD. These immune defects might explain the impaired Th1 responses to NTHi infections of the respiratory tract (29) and the increased susceptibility to bacterial infections in COPD. As Th1-derived IL-2 supports CD8\(^+\) T-cell-dependent cytotoxic infection defenses (15, 53), our data might also help to explain the dysregulation of CD8\(^+\) T cells in COPD (5, 6, 14). A reduced
proliferation rate of activated CD4+ T cells in response to acute infections is not inconsistent with the increased counts of resident T cells that are found in the lungs of subjects with stable COPD because the latter results from insufficient T-cell clearance after infection and/or permanent T-cell recruitment due to bacterial colonization (6, 14).

Despite the stronger reduction (in percent) of IL-2 caused by LPS, absolute IL-2 production in LPS-exposed Th1 cells is still higher in COPD compared with NS and S (Fig. 2C). This is due to the strong increase in baseline IL-2 production of Th1 cells in COPD (Fig. 1B). We were able to reverse the LPS-caused reduction of T-cell counts in COPD by adding recombinant IL-2. This demonstrates that the stronger IL-2 reduction (in percent) caused by LPS is the molecular cause for the stronger reduction of T-cell proliferation in COPD. How can this be explained considering that the absolute IL-2 levels produced by Th1 cells of COPD in the presence of LPS are higher than in NS? IL-2 receptor expression is transient, heterogeneous in different T-cell populations and depends on T-cell receptor activation. T-cell division, in turn, depends on duration and timing of IL-2/IL-2 receptor interaction after T-cell receptor activation and on the degree of saturation of the IL-2/IL-2 receptor system (10). The IL-2/IL-2 receptor system might be saturated by the amounts of IL-2 that are produced by Th1 cells of NS. In support of this, the higher IL-2 levels produced by cells of COPD subjects did not cause an increase in T-cell proliferation. We hypothesize that CD4+ T cells of COPD adapt to the pathologically high IL-2 production during the activation process. After a certain time the IL-2 receptor system would require more IL-2 to become saturated and more IL-2 would be required to maintain a normal proliferation response (comparable to that in NS). This would explain that the stronger IL-2 reduction (in percent) in the presence of LPS causes a stronger reduction of T-cell proliferation independent from the absolute IL-2 levels in COPD. This hypothesis is supported by the data showing that a reversion of the effect of LPS on the counts of T cells in COPD subjects was observed after the addition of recombinant IL-2. Notably, this reversion could only be achieved at concentrations that mirror the average reduction of IL-2 by LPS in COPD but not at lower concentrations.

IL-2 induces IL-2 receptor expression (28). This could be a molecular mechanism involved in the adaption of COPD T cells to the higher IL-2 levels (10). There is evidence that T cells of COPD subjects express more IL-2 receptor than cells of healthy controls (45). A molecular process of adaption with an increase in IL-2 receptor expression would likely require some time to be completed. Following is our interpretation of the data showing that the addition of recombinant IL-2 only at 48 h after T-cell activation reverses the reduction of cell counts by LPS: after 24 h the process is under way with the cells becoming adapted to the sum of endogenously produced and experimentally added IL-2 amounts. Consequently, the effect of LPS on cell counts was not reversed when IL-2 was added 24 h after T-cell activation. After 48 h, the process of adaption is complete and cell counts can be reversed by the addition of recombinant IL-2 at that time. Addition of IL-2 72 h after T-cell activation no longer reversed the LPS effect when cells were counted 24 h later. This indicates that IL-2 requires more than 24 h to influence cell counts. The data further suggest that the suppression of IL-2 by LPS affects the maintenance but not initiation of IL-2-mediated T-cell proliferation.

Th1 cells lack membrane-bound CD14 (24, 44), a coreceptor required for the interaction of TLR4 with canonical smooth LPS (23). Th1 cells primarily respond to truncated LPS forms (short-chain LPS), which CD14 independently activate TLR4 and are synthesized by bacteria relevant in COPD exacerbations, like NTHi (22, 31, 48, 52). The LPS preparations that were used in this study were from Salmonella enteritidis, contained smooth and short-chain LPS, and have been demon-
stratified before to be a suitable model for investigating T-cell responses in COPD (31). Congruently, we demonstrated that NTHI total extract also suppresses IL-2 in Th1 cells and that LPS is the main trigger in this extract.

TLR4 baseline expression in CD4+ non-effector cells and in Th1 cells is not different among NS, S, and COPD. However, LPS induces TLR4 expression in Th1 cells of NS but this effect is significantly reduced in S and COPD (31). We have shown that LPS-induced IFNγ expression is dependent on the upregulation of TLR4 by LPS. Consequently, LPS-induced IFNγ expression is reduced in S and COPD (31). However, the data in Fig. 5B clearly show that TLR4 baseline expression is sufficient for the full IL-2 response to LPS and that the increase in TLR4 levels by LPS exposure does not further enhance this response. This difference might be explained by the time it takes LPS to modulate the expression of these two cytokines: IL-2 is modulated as early as 24 h (Fig. 2A), IFNγ at the earliest 72 h after LPS stimulation (31). Therefore, differences in TLR4 expression cannot explain the difference in IL-2 expression between NS and COPD in the presence of LPS.

Mechanistically, the stronger IL-2 suppression (in percent) by LPS in COPD is caused by an imbalance of the two TLR4 downstream pathways. LPS activates TLR4 to suppress IL-2 in Th1 cells. Upon activation, plasma membrane-located TLR4 binds MyD88, whereas internalized TLR4 on endosomes binds TRIF (25). Both downstream pathways are active in Th1 cells (31), suggesting that the MyD88/TRIF ratio influences whether TRIF (25). Both downstream pathways are active in Th1 cells (31), suggesting that the MyD88/TRIF ratio influences whether TRIF (25). Both downstream pathways are active in Th1 cells (31), suggesting that the MyD88/TRIF ratio influences whether TRIF (25). Both downstream pathways are active in Th1 cells (31), suggesting that the MyD88/TRIF ratio influences whether TRIF (25). Both downstream pathways are active in Th1 cells (31), suggesting that the MyD88/TRIF ratio influences whether TRIF (25). Both downstream pathways are active in Th1 cells (31), suggesting that the MyD88/TRIF ratio influences whether TRIF (25). Both downstream pathways are active in Th1 cells (31), suggesting that the MyD88/TRIF ratio influences whether TRIF (25). Both downstream pathways are active in Th1 cells (31), suggesting that the MyD88/TRIF ratio influences whether TRIF (25). Both downstream pathways are active in Th1 cells (31), suggesting that the MyD88/TRIF ratio influences whether...
IKKe/TBK signaling at the expense of MyD88/IRAK signaling in response to LPS (Fig. 10). Activation of T-cell receptors induces IL-2 transcription, which can be repressed by LPS via both, TLR4/MyD88 and TLR4/TRIF signaling. Whereas the IL-2 gene is primarily repressed by MyD88/IRAK signaling in NS, TRIF/TBK/IKKe signaling blocks the IL-2 gene in COPD (Fig. 10). The stronger suppression (in percent) of IL-2 by LPS in COPD is time dependent: there is no difference between NS and COPD after 24 and 48 h but after 72 h of LPS exposure. LPS has been shown to induce immediate and short-term cytokine responses via the MyD88 pathway but long-term cytokine responses via the TRIF pathway (8, 42). This means that the MyD88 pathway becomes inactivated earlier than the TRIF pathway by negative feedback mechanisms. This can explain the stronger effect of LPS on IL-2 over time in COPD.

The interpretation of the results relies in part on the use of chemical protein kinase inhibitors and blocking peptides for the two pathways, which might be a limitation of the study. Particularly, chemical inhibitors are not 100% specific for one protein kinase and might also hit related kinases, although usually at significantly higher IC₅₀ values. To our knowledge the IRAK-1/-4 inhibitor and BX795 are the most specific inhibitors for IRAK-1/-4 or IKKe/TBK1, respectively, currently available. They have high selectivity scores and/or lowest IC₅₀ values compared with multiple other tested kinases (2, 4, 43). Nonetheless, there is evidence that BX795 might efficiently block three other protein kinases even at the low concentration used in this study (4). To minimize the probability of false interpretations, we used not only a chemical inhibitor that blocks the key protein kinase but also a peptide inhibitor that blocks the upstream adapter molecule of a pathway. This was done for each pathway in parallel experiments. We found the data to be congruent. This strongly supports our interpretation of the results discussed above.

Endogenous MyD88/IRAK pathway antagonists like IRAK-M are upregulated in Th1 cells of COPD (31).
might contribute to the pathway switch from MyD88 to TRIF signaling in COPD. However, the upregulation of IRAK-M is related to pack years, is also seen in cells of current smokers without airway obstruction, and can explain the reduced IFNγ production in response to LPS, which is also smoking dependent in COPD (31): LPS signals via the TRIF pathway to activate the IFNγ gene but this requires the upregulation of TLR4 by LPS, which is mediated by the MyD88 pathway. The pathological upregulation of the MyD88 pathway antagonists impairs the upregulation of TLR4 and consequently the production of IFNγ by LPS (31). However, stronger suppression of IL-2 and Th1-cell proliferation by LPS was observed in current smokers with COPD but not in current smokers without COPD and, hence, cannot satisfactorily be explained by the smoking-dependent upregulation of MyD88/IRAK antagonists. As the MyD88/TRIF imbalance was smoking independently established in COPD, we conclude that this molecular pathology is additionally required to cause the stronger suppression of IL-2 and Th1-cell proliferation in response to LPS in COPD (Fig. 10). Summarized, the systemic suppression of MyD88/IRAK and the upregulation of TRIF signaling due to multiple molecular pathologies impair T-cell responses to acute bacterial infections in COPD.

Both molecular pathologies that have been discovered in activated T cells of COPD in this study, the IL-2 hyperproduction and the MyD88/TRIF imbalance, are independent from smoking. If not smoking, which COPD features could cause these molecular pathologies? COPD is associated with systemic inflammation, which is characterized by elevated concentrations of various soluble inflammatory proteins in the peripheral blood (19). The repeated or permanent exposure to these inflammatory proteins might cause molecular changes in circulating T cells. We were able to provide some experimental support for this hypothesis: we aimed at mimicking the environment of circulating T cells in COPD by culturing them in medium supplemented with serum obtained from COPD subjects. In these conditions, CD4⁺ T cells of NS produced more IL-2 and expressed less MyD88 compared with the cultivation in medium supplemented with serum of S or NS. However, TRIF expression in CD4⁺ T cells of NS was not influenced by the different sera. Nonetheless, constituents of COPD serum are able to induce a part of the molecular pathologies in CD4⁺ T cells of NS characteristic for COPD. This supports the hypothesis that systemic inflammation causes the molecular defects in CD4⁺ T cells of COPD. However, if so, why was the upregulation of TRIF not reflected in these experimental settings? Incubation of purified T cells in serum of COPD subjects is a suitable experimental model for mimicking a COPD-characteristic systemic inflammatory environment. However, it is limited in time and there are no other blood cells present, some of which are also increased in COPD compared with healthy subjects (15). Therefore, TRIF upregulation in non-effector CD4⁺ T cells in COPD might require a longer exposure to the inflammatory proteins in the circulation or might be a response to specific blood cells, e.g., via interaction of T cells with nonsoluble surface molecules of other leukocytes.

These data raise the question of candidate molecules associated with systemic inflammation in COPD that induce the molecular pathologies in CD4⁺ T cells. Cigarette smoking might induce systemic inflammation in subjects that have not (yet) developed a COPD (20). However, the molecular defects were observed in CD4⁺ T cells of COPD subjects but not in those of current smokers without COPD. Therefore, the concentrations of candidate proteins should be higher in serum of COPD subjects not only compared with nonsmoking healthy subjects but also to current smokers without COPD and/or associated with disease progression. Accordingly, candidate soluble proteins are TNFα, IL-6, and CCL-18, for example (11, 19, 50). TNFα might be particularly interesting as it has been shown to induce TRIF expression (12). However, preincubation with these candidate proteins in our cell culture system did not cause molecular pathologies characteristic for COPD, IL-2 upregulation, or MyD88/TRIF imbalance in ex vivo-generated CD4⁺ T cells of NS (J. Knobloch, unpublished data). We conclude that the causes for the molecular T-cell defects in COPD are associated with systemic inflammation but that they are more complex than a simple response to a single inflammatory mediator.

Our data suggest an impaired defense against acute bacterial airway infections in COPD due to a reduced IL-2-dependent T-cell proliferation in the presence of LPS. Targeting this immunologic pathology in therapy might reduce airway inflammation and exacerbation rates and/or periods. Therefore, we aimed at reversing it in cell culture. p38 MAPK is an IL-2 gatekeeper (33 and this study), is downstream of both TLR4/MyD88 and TLR4/TRIF signaling, and, therefore, regulates IL-2 inhibition by LPS in Th1 cells independently from disease. Consequently, p38 MAPK was an auspicious molecular target. Moxifloxacin blocks p38 MAPK (49 and this study). The closely related fluoroquinolone ciprofloxacin upregulates IL-2 in CD4⁺ T cells (16). Therefore, moxifloxacin was an auspicious candidate drug. Indeed, moxifloxacin reversed the LPS-caused suppression of IL-2 and Th1-cell counts in COPD. Moxifloxacin is used to treat bacterial infections in COPD and reduces exacerbation rates (40). Our data suggest that moxifloxacin could cause a recovery of antigen-specific Th1-cell numbers in infection defense, which might contribute to its efficacy. The immunomodulatory properties of moxifloxacin are believed to explain its superiority to macrolides in eradicating NTHi in exacerbated COPD (40). In contrast to moxifloxacin, macrolides suppress IL-2 in activated T cells (26). Therefore, our data might also help to explain the superiority of moxifloxacin over macrolides in eradicating NTHi in COPD exacerbations. In response to airway infections, recruited T cells primarily proliferate in the draining lymph nodes of the lung (51). Thus our study might provide first arguments in support of a utility of inhaled moxifloxacin with regard to improving infection defense in COPD by recovering T-cell counts. This might reduce exacerbation rates. However, despite the putative beneficial effects on T-cell proliferation, IL-2 upregulation by moxifloxacin might enhance airway inflammation.

Th1 cells are crucial for immune responses to bacterial infections and our data provide evidence for an insufficient Th1-based immune response to infections with gram-negative bacteria. However, gram-positive bacteria can also trigger exacerbations and their PAMPs are primarily lipopeptides that bind to TLR2/TLR1 or TLR2/TLR6 heterodimers, which activate downstream Myd88 but not TRIF signaling (8, 42). Th1 cells express TLR2 and respond to bacterial lipopeptides with an increase in IFNγ production and proliferation (34). Neither IL-2 expression nor the influence of smoking or COPD patho-
genesis has been investigated in this context. However, there are data from another T-cell subset available, from Th2 cells, which are often increased in exacerbated COPD. This indicates that they are also associated with infection defense mechanisms. Th2 cells also produce IL-2, albeit at a lower level than Th1 cells. Coculturing ex vivo-generated Th2 cells with accessory cells (e.g., with monocytes) increases IL-2 production. Compared with healthy nonsmokers, cocultured CD4+ and CD8+ T cells of COPD subjects and current smokers without airway obstruction produce more IL-2 upon activation towards Th2 in the presence of accessory cells (38). Consequences on T-cell proliferation were not investigated in this study. Therefore, besides Th1-derived also Th2-derived IL-2 might contribute to the increase in inflammation in exacerbated COPD.

Lipoteichoic acid (LTA), another PAMP of gram-positive bacteria, which might signal via TLR2 (47), increases IL-2 production of Th2 cells. However, LTA requires the presence of accessory cells, particularly monocytes, to do so (38). Therefore, this is rather an indirect response of Th2 cells to LTA mediated by monocytes and other accessory cells than a response to a direct stimulation of TLR2 on the surface of Th2 cells by LTA. This is in contrast to the direct suppression of IL-2 in Th1 cells by LPS/TLR4 signaling shown here. In CD8+ T-cell subpopulations and in total T cell preparations (which contain CD4+ and CD8+ T cells), LTA suppresses IL-2 under Th2-polarizing conditions in the presence of accessory cells but without differences between COPD and healthy subjects (38). We conclude that it depends on T-cell subtypes, on the presence of accessory cells, and on the pattern of TLR ligands whether a bacterium increases or suppresses IL-2 production in T cells. Whereas the effects of LTA on IL-2 might be indirect, mediated by accessory cells, and not be influenced by COPD pathogenesis, the effects of LPS are direct and stronger in COPD.

Summarized, we have demonstrated two molecular pathologies in circulating CD4+ T cells of COPD subjects. 1) They smoking independently release increased IL-2 amounts upon activation towards Th1. 2) They have a defect in TLR downstream signaling, which results in an impaired IL-2-dependent T-cell proliferation in response to acute bacterial infections of the lung (or other organs). This might contribute to airway inflammation and might help to explain the impaired infection defense and the increased susceptibility to bacterial infections in COPD. Targeting TLR4 signaling or p38 MAPK in Th1 cells, e.g., with moxifloxacin, might reverse this immunologic pathology and reduce exacerbation rates.

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AUTHOR CONTRIBUTIONS

J.K., J.R., and A.K. conception and design of research; J.K., S.-J.C., and A.K. performed experiments; J.K., S.-J.C., and D.J. interpreted results of experiments; J.K. prepared figures; J.K. drafted manuscript; J.K., S.-J.C., S.Y., J.R., D.J., and A.K. approved final version of manuscript; S.-J.C., D.J., and A.K. edited and revised manuscript.

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