Chlamydophila pneumoniae phospholipase D (CpPLD) drives Th17 inflammation in human atherosclerosis

Marisa Benagiano[a,b,1], Fabio Munari[c,1], Alessandra Ciervo[d], Amedeo Amedei[a,b], Silvia Rossi Paccani[e], Fabiola Mancini[d], Mauro Ferrari[f], Chiara Della Bella[a,b], Camilla Ulivi[b], Sofia D'Elios[b], Cosima T. Baldari[e], Domenico Prisco[a,g], Marina de Bernard[c,h,2], and Mario M. D'Elios[a,b,2]

[a]Patologia Medica, Azienda Ospedaliero-Universitaria Careggi Firenze, 50134 Florence, Italy; Departments of [b]Internal Medicine and [g]Critical Care Medicine, University of Florence, 50134 Florence, Italy; [c]Venetian Institute of Molecular Medicine Padua, 35129 Padua, Italy; [d]Istituto Superiore Sanità, 00161 Rome, Italy; [e]Department of Evolutionary Biology, University of Siena, 53100 Siena, Italy; [f]Vascular Surgery Pisa, University of Pisa, 56124 Pisa, Italy; and [h]Department of Biology, University of Padua, 35121 Padua, Italy

Edited* by Brigitte A. Askonas, Imperial College London, London, United Kingdom, and approved December 7, 2011 (received for review July 21, 2011)

Phospholipases are produced from bacterial pathogens causing very different diseases. One of the most intriguing aspects of phospholipases is their potential to interfere with cellular signaling cascades and to modulate the host–immune response. Here, we investigated the role of the innate and acquired immune responses elicited by *Chlamydophila pneumoniae* phospholipase D (CpPLD) in the pathogenesis of atherosclerosis. We evaluated the cytokine and chemokine production induced by CpPLD in healthy donors' monocytes and in vivo activated T cells specific for CpPLD that infiltrate atherosclerotic lesions of patients with *C. pneumoniae* antibodies. We also examined the helper function of CpPLD-specific T cells for monocyte matrix metalloproteinase (MMP)-9 and tissue factor (TF) production as well as the CpPLD-induced chemokine expression by human venular endothelial cells (HUVECs). We report here that CpPLD is a TLR4 agonist able to induce the expression of IL-23, IL-6, IL-1β, TGF-β, and CCL-20 in monocytes, as well as CXCL-9, CCL-20, CCL-4, CCL-2, ICAM-1, and VCAM-1 in HUVECs. Plaque-derived T cells produce IL-17 in response to CpPLD. Moreover, CpPLD-specific $CD4^+$ T lymphocytes display helper function for monocyte MMP-9 and TF production. CpPLD promotes Th17 cell migration through the induction of chemokine secretion and adhesion molecule expression on endothelial cells. These findings indicate that CpPLD is able to drive the expression of IL-23, IL-6, IL-1β, TGF-β, and CCL-20 by monocytes and to elicit a Th17 immune response that plays a key role in the genesis of atherosclerosis.

cardiovascular diseases | inflammation | thrombosis | stroke | vaccine

Host–pathogen interactions can lead to different types of outcomes depending on the kind of relationship that is established when a microorganism enters his host cell. One of the major factors involved in shaping "the picture" is the priming of the immune system that can lead either to an effective clearance of the pathogen or to an evasion of the immune response. In the last decades, many efforts have been made to identify microbial products that can have a key role in the pathogenesis of infections by activating, modulating or down-regulating the host defenses. Phospholipases (PLs) are a heterogeneous group of proteins with enzymatic activity able to hydrolize phospholipids (1). They are produced by both procaryotic and eukaryotic organisms and are likely to be involved in membrane disruption occurring host cell invasion. The realization that some fungal and bacterial toxins were secreted PLs indicated that these molecules were, in fact, virulence factors (2). Each enzyme is able to cleave a specific ester bond on the phospholipid substrate, which forms the basis of their nomenclature. PLD was first described in the early '50s as a distinct enzyme with a specific phosphodiesterase activity leading to the hydrolisis of phosphatidilcholine into phosphatidic acid (PA) and choline (3). PA acts as a second messenger molecule and is involved in key cellular processes such as membrane trafficking, actin cytoskeleton remodelling, cell proliferation, and cell survival. *Chlamydophila pneumoniae* is an obligate intracellular bacterium present as free infectious, nonmetabolic elementary bodies (EB) that transmit infection to susceptible eukaryotic cells such as epithelial and endothelial cells, smooth muscle cells, and macrophages. During its developmental cycle, *C. pneumoniae* differentiates into metabolically active reticular bodies (RB) able to replicate by binary fission and differentiate into EB, which cause host cell lysis, thereby progressing the infectious cycle (4). Productive infection is not the only possible outcome of *C. pneumoniae* interaction with host cells. Persistence due to nutritional deprivation, antibiotic treatment, or immune reaction leads to a chronic or latent infection characterized by the presence of abnormal RB that fail to mature (5). Some aspects of the developmental cycle of *C. pneumoniae* suggest a direct implication of PLD in pathogenesis, specifically by affecting the regulation of lipid metabolism and lipid exchange between *C. pneumoniae* and host cells. Over the past two decades a strong link between chronic infection by *C. pneumoniae* and the inflammatory process in atherosclerosis has also been suggested (6). To characterize the activity of PLD as virulence factor in *C. pneumoniae* infection, we have studied the effect of *C. pneumoniae* PLD (CpPLD) (7) on the ability of monocytes to prime the innate immune response and on $CD4^+$ T cells to develop an adaptive immune response. In addition we analyzed the possible role of CpPLD on endothelial cell (EC) activation. We have found that CpPLD-specific T cells were present within the lesion and they exhibited a Th17 cytokine pattern. Moreover, CpPLD-specific T cells were able to strongly promote the production of both tissue factor (TF) and metalloproteinase 9 (MMP-9) involved in plaque rupture and atherothrombotic events. We demonstrated that CpPLD was able to activate monocytes by binding Toll-like receptor 4 (TLR4), leading to the production of IL-23, IL-6, IL-1β, TGF-β, and CCL-20, molecules critical for the generation, differentiation, and maintenance of Th17 cells. Furthermore, CpPLD was able to elicit the up-regulation of the expression of several chemokines and adhesion molecules such as CXCL-9, CCL-20, CCL-4, CCL-2, ICAM-1, and VCAM-1 in human venular endothelial cells (HUVECs). These results demonstrate that CpPLD is able to drive Th17 inflammation within the atherosclerotic plaque and suggest that PLD-activated Th17 cells play a crucial role in atherosclerosis, plaque rupture, and atherothrombotic events.

Author contributions: M.B., M.d.B., and M.M.D. designed research; M.B., F. Munari, A.C., A.A., S.R.P., F. Mancini, M.F., C.D.B., C.U., S.D., C.T.B., and D.P. performed research; M.B., F. Munari, C.T.B., M.d.B., and M.M.D. analyzed data; and M.B., M.d.B., and M.M.D. wrote the paper.

The authors declare no conflict of interest.

*This Direct Submission article had a prearranged editor.

[1]M.B. and F. Munari contributed equally to this work.

[2]To whom correspondence may be addressed. E-mail: delios@unifi.it or marina.debernard@unipd.it.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1111833109/-/DCSupplemental.

Results

PLD Drives IL-17 and IL-21 Secretion from Th Cells Derived from Atherosclerotic Plaques. T-cell lines from atherosclerotic plaques were cultured and analyzed for IL-17 and IL-21 production in culture supernatants. After 72 h of stimulation with immobilized anti-CD3 mAb, they produced high levels of IL-17 and IL-21 (Fig. 1*A*). We next examined whether carotid plaque-derived T cells from patients with atherosclerotic arteriopathy produced IL-17. Atherosclerotic plaque-derived anti-CD3 mAb-induced T-cell lines were expanded from seven *Cp-positive* (*Cp-pos*) and from five *Cp-negative* (*Cp-neg*) patients as control by addition of human recombinant IL-2 (hrIL-2) every 3 d. At day 15, T-cell blasts of each plaque T-cell line were stimulated with CpPLD, tetanus toxoid (TT), or purified protein derivative (PPD) in the presence of autologous antigen presenting cells (APCs) for 48 h in ELISPOT microplates coated with anti–IL-17 antibody. At the end of the culture period, the number of IL-17 spot-forming cells (SFCs) were counted (Fig. 1*B*). A significant proportion of all plaque-derived T-cell lines, under MHC restricted conditions, reacted specifically to CpPLD by producing IL-17 in a dose-dependent fashion (Fig. 1*C*).

PLD-Specific T-Cell Clones Infiltrating the Atherosclerotic Lesions of *C. pneumoniae*-Positive Patients Display a Th17 Phenotype. In vivo-activated plaque-infiltrating T cells were expanded in vitro in hrIL-2–conditioned medium, subsequently cloned and studied for their phenotypic and functional profile (8).

A total number of 223 $CD4^+$ and 49 $CD8^+$ T-cell clones were obtained from plaques of the seven *Cp-pos* patients. For each patient, $CD4^+$ and $CD8^+$ plaque-derived T-cell clones were assayed for proliferation in response to medium, *C. pneumoniae* EB (CpEB), CpPLD, *H. pylori* lysate (NCTC11637 strain; 10 μg/mL) and PHA. None of the $CD8^+$ T-cell clones showed proliferation to CpPLD, CpEB, and to *H. pylori* lysate. In contrast, 44 (20%) and 13 (6%) of the 223 $CD4^+$ T-cell clones generated from plaque-infiltrating T cells proliferated significantly to CpEB and to CpPLD, respectively, but not to the *H. pylori* lysate (Fig. 2 and Tables S1 and S2). Upon antigen stimulation with CpPLD, all of the 13 PLD-specific T-cell clones secreted IL-17. Among the IL-17–secreting PLD-specific T-cell clones, five were producing both IL-17 and IFN-γ (Fig. S1*A*). None of these clones where able to produce IL-4, whereas all clones were able to produce IL-21 in response to antigen stimulation (Fig. S1*B*). A total number of 165 $CD4^+$ and 32 $CD8^+$ T-cell clones were obtained from plaques of five *Cp-neg* patients. None of these clones proliferated to either *C. pneumoniae* or *H. pylori* (Table S1).

PLD-Specific Plaque-Infiltrating T Cells Promote TF and MMP-9 Production by Monocytes. Studies of human vessels suggest that gelatinases in general are highly expressed in fatty streaks and atherosclerotic plaques compared with normal regions of the vessel (9). Because plaque rupture and thrombosis are notable complications of atherosclerosis, we asked whether plaque-infiltrating PLD-specific T cells had the potential to express helper function for TF and MMP-9 production by monocytes. Antigen-stimulated PLD-specific T-cell clones from plaques were cocultured with autologous monocytes, and the levels of TF and MMP-9 were measured. Antigen stimulation resulted in the expression of substantial help for TF production by monocytes (Fig. 3*A*). Likewise monocytes incubated with antigen-stimulated CpPLD-specific T-cell clones were able to produce significant levels of MMP-9 (Fig. 3*B*).

PLD Activates Monocytes to Release Cytokines Essential for the Differentiation of Th17 Cells. IL-23 is a heterodimeric cytokine sharing the p40 subunit with IL-12 and having a distinct p19 subunit (10). CpPLD was able to induce the expression of both IL-12p40 and IL-23p19 in monocytes (Fig. 4 *A* and *B*). The kinetics of expression of the two subunits were consistent with those of protein accumulation in culture supernatants (Fig. 4*C*).

IL-23 is an essential survival factor for Th17 cells, because these cells are not present in mice lacking IL-23 (11). Recent

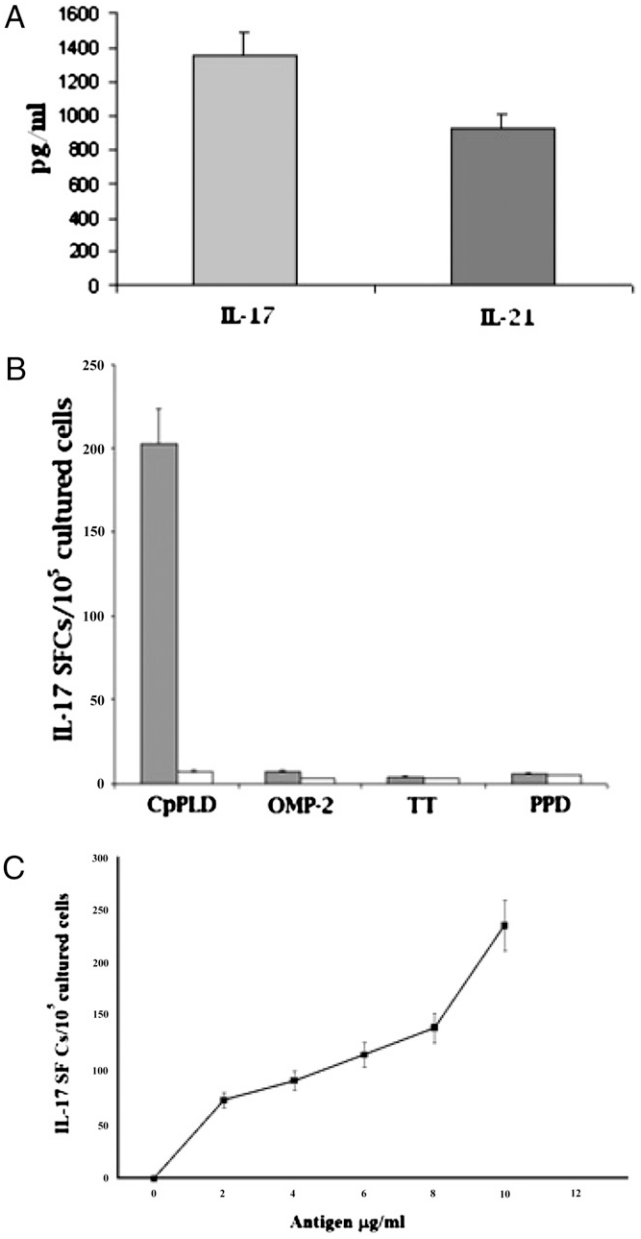

Fig. 1. CpPLD-driven IL-17 secretion by atherosclerotic plaque Th cells from patients with atherosclerosis. (*A*) Release of IL-17 and IL-21 from fresh atherosclerotic plaque Th cells after 72 h of stimulation with immobilized anti-CD3 monoclonal antibody (mAb). (*B*) Numbers of IL-17 SFCs after stimulation of fresh atherosclerotic plaque Th cells with CpPLD, OMP-2, TT, or PPD. Atherosclerotic plaque-derived anti-CD3 mAb-induced T-cell lines were expanded from *Cp-pos* (gray bars) and from *Cp-neg* (white bars) patients as control by addition of hrIL-2, every 3 d. At day 15, T-cell blasts from each line were stimulated for 48 h with CpPLD, OMP-2, TT, or PPD in the presence of irradiated autologous APCs in enzyme-linked immunospot microplates coated with anti–IL-17 antibody. IL-17 spot-forming cells were then counted by using an automated reader. After specific stimulation with CpPLD, a significant proportion of *Cp-pos* atherosclerotic plaque-derived Th cells produced IL-17. (*C*) Dose–response effect of graded concentrations of CpPLD antigen on the numbers of IL-17 spot-forming cells. Values are the mean ± SD number of spot-forming cells per 10^5 cultured cells over background levels.

IMMUNOLOGY

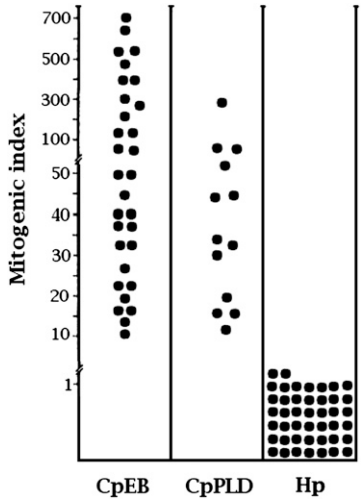

Fig. 2. Antigen specificity of plaque-infiltrating T-cell clones. Th clones were tested for proliferation to (CpEB), recombinant CpPLD, and *H. pylori* lysate in the presence of irradiated autologous APCs.

findings suggest, however, that IL-23 is not required during the early differentiation of Th17 cells, because IL-23R is not expressed on naïve T cells, but would serve to expand previously differentiated Th17 cells (12). Other factors such as IL-6, IL-21, and TGF-β involved in the initial differentiation of Th17 were identified (13). Accordingly, the messenger RNA (mRNA) and protein amounts of these cytokines were determined after CpPLD stimulation of monocytes. Increased levels of IL-6 mRNA were found, with a peak production after 5 h of stimulation, and high levels of IL-6 were measured in culture supernatants (Fig. 5 *A* and *B*). CpPLD also induced high expression of TGF-β mRNA, which started to increase and peaked after 1 h from CpPLD addition, leading to the accumulation of the protein in the supernatant (Fig. 5 *C* and *D*). IL-1β mRNA was found to significantly increase after 5 h following CpPLD stimulus and peaked after 24 h from CpPLD addition along with the protein production in the supernatants (Fig. 5 *E* and *F*). Furthermore, CpPLD was able to stimulate CCL-20 mRNA production, which started to increase and peaked after 5 h from CpPLD addition and was still substantial after 24 h from stimulation with high levels of protein production detectable in the supernatants (Fig. 5 *G* and *H*). These findings indicate that CpPLD, acting on monocytes, contributes to create a cytokine milieu enriched in IL-6, TGF-β, IL-1β, and IL-23 with a strong potential of driving the T-cell differentiation toward the Th17 subset (Fig. S3). Indeed, the up-regulation of CCL-20 mRNA production is a significant hallmark for the presence and maintenance of CCR6-positive Th17 cells through their positive chemotactic stimulus.

IL-23 Secretion Follows PLD Binding to TLR4. To investigate the mechanism of CpPLD monocyte activation, we evaluated the possibility that CpPLD activates a TLR-mediated signaling. Considering that bacterial proteins preferentially activate monocytes and dendritic cells via TLR2 or TLR4, we used human embryonic kidney (HEK) 293 cells transfected with plasmids encoding either of them. HEK293 cell lines lack expression of endogenous TLRs, although their TLR signaling machinery is fully functional. The common pathway leading to NF-κB activation requires phosphorylation and degradation of the cytosolic inhibitor of NF-κB, IκB-α. The engagement of a specific TLR, expressed on HEK293, was monitored by evaluating the phosphorylation of IκB-α after exposure to CpPLD. As shown in Figs. S4 and S5, phosphorylation of IκB-α was observed in cells expressing TLR4, but not in those expressing TLR2. In addition, an anti-TLR4 blocking antibody abrogated the PLD induction of IL-23 (Fig. S4).

PLD Promotes Th17 Cells Migration Through the Induction of Chemokine Production and Adhesion Molecule Expression on HUVEC. Chemokines and adhesion molecules produced by ECs are important in driving T-cell trafficking. Given that CCL-4, CCL-2, CCL-20, and CXCL-9 acting on CCR5, CCR2, CCR6, and CXCR3, respectively, are able to chemoattract Th17 cells (14), we investigated whether CpPLD was able to induce mRNA expression and protein production of different chemokines and adhesion molecules in HUVECs. As shown in Fig. S6, upon stimulation with CpPLD HUVEC were able to produce significant levels of CCL-4, CCL-2, CCL-20, CXCL9, sICAM-1, and sVCAM-1.

Discussion

One of the most intriguing aspects of PLs as virulence factors is their potential ability to modulate host–immune response. Little is known about CpPLD, and additional data are needed to provide an understanding of the common biological processes required for infection and survival of this pathogen in mammalian cells. In this study, we investigated the type of innate and acquired immune responses evoked by CpPLD in *Cp-pos* patients with atherosclerosis. A serological response to CpPLD has been described in the sera of patients with acute coronary syndromes infected by *C. pneumoniae* but not in those of healthy

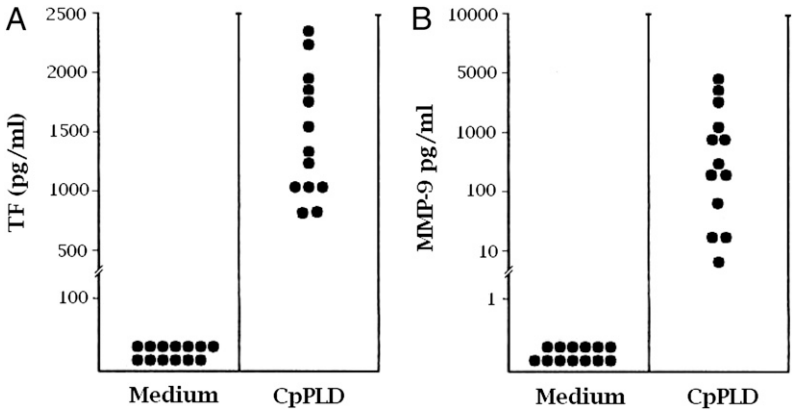

Fig. 3. CpPLD-specific T cells induce TF and MMP-9 production by autologus monocytes. To assess their ability to induce TF and MMP-9 production by autologus monocytes, CpPLD-specific Th clones were cocultured with autologous monocytes in the presence of medium or CpPLD. TF (*A*) and MMP-9 (*B*) production by monocytes were assessed by ELISA. The results shown represent the TF and MMP9 levels induced by T-cell clones over the TF and MMP9 production in cultures of monocytes alone, respectively.

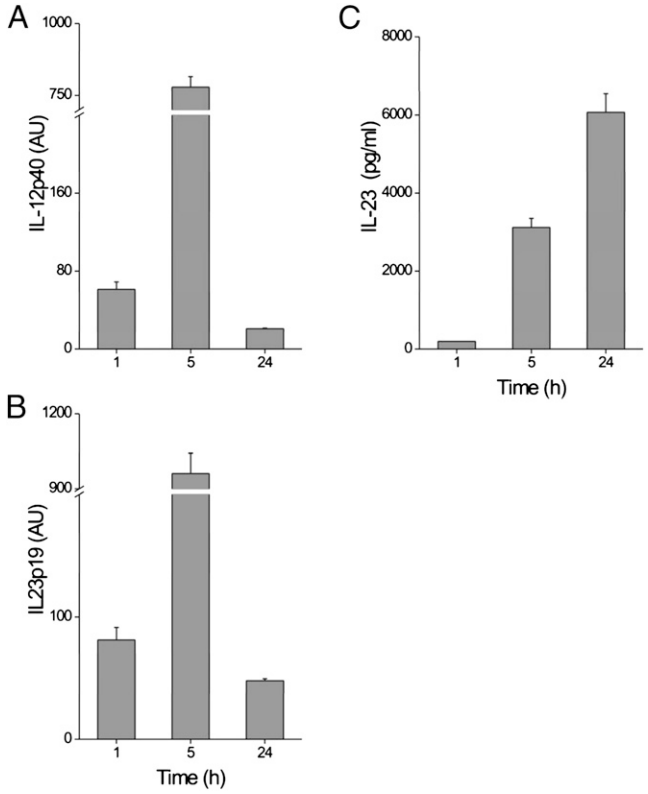

Fig. 4. Kinetics of the synthesis and production of interleukin-23 (IL-23) by freshly isolated monocytes stimulated with CpPLD. Levels of IL-12p40 (*A*) and IL-23p19 (*B*) cytokine mRNA in monocytes from healthy donors were determined at the indicated time points after CpPLD stimulation, by quantitative real-time PCR analysis. Shown are representative results from one of four experiments conducted with different cell preparations. Values are the mean and SD arbitrary units (AU). Levels of IL-23 protein in monocytes from healthy donors were determined at the indicated time points after CpPLD stimulation by ELISA of culture supernatants from the same cells that had been harvested for mRNA evaluation (*C*). The kinetics of production were comparable among the different experiments, whereas the amounts produced varied among the different donors. Values are the mean ± SD of four independent experiments by using triplicate samples.

subjects and anti-Cp antibody titers were higher in subjects with both IgG and IgA than in those with IgG only (15). T cells play an important role in the genesis of atherosclerosis that has been defined a Th1-driven immunopathology (16, 17), and we have demonstrated that Th1 cells, producing high levels of IFN-γ, are crucial for the development of the disease (18, 19). Given that atherosclerosis can occur and progress even in IFN-γ–deficient mice or its receptor, although with a lower lesion burden (20), other Th cells and factors are presumably involved in the genesis of the atheroma. Recently, a third subset of effector Th cells, namely Th17, has been discovered (21). Th17 cells are potent inducers of tissue inflammation and have been associated with the pathogenesis of many experimental autoimmune diseases and human inflammatory conditions (22, 23). In the lymphocytic infiltrates of human atherosclerotic plaques, we have found the presence of in vivo-activated plaque-infiltrating T cells able to produce IL-17 and IL-21 in response to CpPLD. Among the clonal progeny of T cells infiltrating the lesions, we demonstrated the presence of CpPLD-specific T cells able to secrete IL-17. A significant number (38%) of IL-17–producing T cells are also IFN-γ producers. This finding is in agreement with a previous report that demonstrated the concomitant production of IL-17 and IFN-γ by human coronary artery-infiltrating T cells (24, 25). Plaque rupture and thrombosis are notable complications of atherosclerosis (26). The CpPLD-specific T-cell clones revealed their ability to induce macrophage production of TF upon antigen stimulation. Several studies have provided a clear indication of an important role of MMPs in atherothrombosis (9). It has been suggested that the myocardial or cerebral ischemic complications may, in fact, depend more on the plaque composition than on the degree of stenosis. The composition of the extracellular matrix may affect plaque progression and also be a key determinant of plaque vulnerability (27). The infiltration of inflammatory cells into the atherosclerotic lesion results in a major increase of MMP activity (9). It is of note that CpPLD-specific T-cell clones were able to stimulate the production of MMP-9 from the monocytes of patients. Th17 cells were shown to play a key role in experimental mouse models of atherosclerosis; IL-17 is proatherogenic in both diet-induced atheroma development and *C. pneumoniae* infection-mediated acceleration of atherosclerotic lesions in the presence of high fat diet (HFD) in mice (28). In fact, in IL-17$^{-/-}$ mice fed with HFD and infected with *C. pneumoniae,* the aortic lesion size and lipid composition as well as macrophage accumulation in the plaques were significantly diminished, and the progression of the process was significantly reduced compared with WT mice. *C. pneumoniae* was known to induce IL-17 (25), but the nature of the bacterial molecule(s) able to stimulate Th17 development was not known. We have demonstrated that CpPLD was able to activate both the mRNA expression and the production of IL-23, IL-6, IL-1β, and TGF-β by monocytes. All these cytokines have proved to be crucial for the induction of Th17 cell responses (11). These findings do not exclude the possibility that additional bacterial factors contribute to the generation of the Th17 cell response found in the atherosclerotic lesion. Furthermore, CpPLD was also able to induce the up-regulation of both mRNA and protein production of CCL-20 in monocytes, a ligand for CCR6 expressed by activated Th17 cells (29). Th17 cells are highly heterogeneous in terms of trafficking receptors. Many of these receptors are involved in T-cell migration into inflamed tissue sites; therefore, the receptor expression profile of Th17 cells is consistent with their inflammatory activities in non-lymphoid tissues (14). We also demonstrated that CpPLD is a TLR-4 agonist that was able to activate NF-κB in TLR4-transfected but not in TLR2-transfected HEK 293 cells. The involvement of the TLR4 receptor in the activation of monocytes by CpPLD was further supported by the abrogation of CpPLD-induced IL-23 expression by a specific anti-TLR4 blocking antibody. Current evidence indicates that CCL-4, CCL-2, CCL-20, CXCL-9, VCAM-1, and ICAM-1 are important in the genesis of atherosclerosis (30) and that CCL2 and CCL20 are relevant in promoting Th17 cell migration (31–33). We have investigated the potential role of CpPLD on the expression of different molecules by EC. Our data obtained in HUVECs demonstrated that CpPLD was able to induce an increased expression of different chemokines such as CXCL-9, CCL-20, CCL-4, and CCL-2. Interestingly, this chemokine profile suggests that Th17 cells selectively migrate within the atherosclerotic lesion upon CpPLD stimulation. Such a chemokine pattern can also account for the presence of CpPLD-specific T cells able to produce both IL-17 and IFN-γ, which share a chemokine receptor phenotype associated with both Th17 and Th1 cells (14, 25). Leukocyte interactions with vascular endothelium at sites of inflammation can be dynamically regulated by activation-dependent adhesion molecules. We investigated the role of CpPLD in the expression of ECs adhesion molecules involved in the adhesion and diapedesis of leukocytes. Our results obtained on HUVECs demonstrated that CpPLD was able to up-regulate the mRNA expression of sICAM-1 and sVCAM-1, thus promoting tissue inflammation within the plaque. Overall, our findings support the concept that a crucial component of atherosclerosis is represented by T-cell–mediated immunity and that chronic Th response against

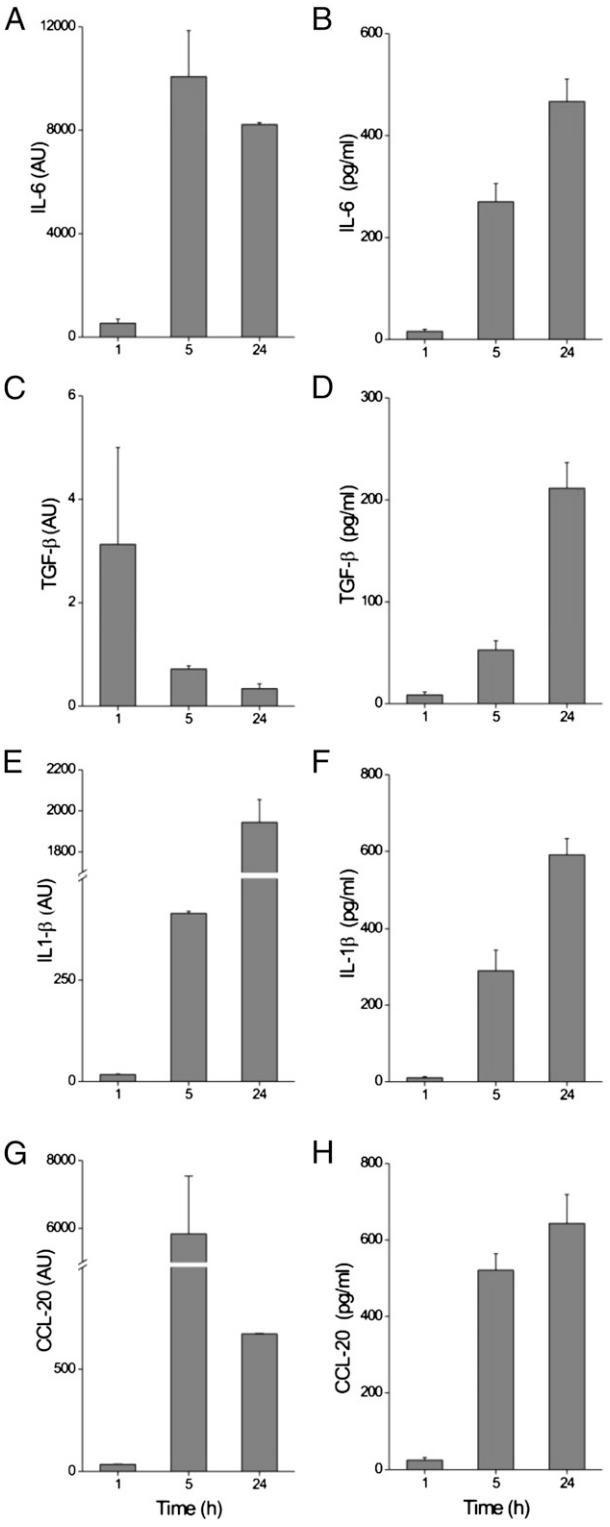

Fig. 5. Kinetics of the synthesis and production of IL-6, TGF-β, IL-1β, and CCL-20 by freshly isolated monocytes stimulated with CpPLD. (*A, C, E,* and *G*) Levels of IL-6, TGF-β IL-1β, and CCL-20 mRNAs were determined by quantitative real-time PCR at the indicated time points after CpPLD stimulation. AU, arbitrary units. (*B, D, F,* and *H*) Levels of IL-6, IL-1β, and CCL-20 proteins were determined by ELISA at the indicated time points after CpPLD stimulation. TGF-β content was measured by using CCL64 bioassay. Culture supernatants from the same monocytes that were harvested for mRNA evaluation were used for these analyses. Values are the mean and SD of four independent experiments by using triplicate samples.

C. pneumoniae plays an important role in the genesis of atheroma. Specifically, we have demonstrated that CpPLD is able to elicit a Th17 immune response within the plaque by activating ECs and macrophages to express and secrete a wide variety of chemokines and cytokines. Within CpPLD-specific IL-17–producing Th cells, the majority were polarized Th17 cells, whereas others were able to produce both IFN-γ and IL-17. Thus, it is possible to speculate that Th17 and Th1 cells comigrate to the inflamed tissue and cooperate to the ongoing inflammatory process within the atherosclerotic lesion (21). Our results demonstrate that CpPLD is a major *C. pneumoniae* factor able to drive Th17 inflammatory process in atherosclerosis and suggest that Th17 cell pathway and CpPLD may represent unique therapeutic targets for the prevention and treatment of the disease.

Materials and Methods

Reagents. hrIL-2, TT, and PPD were provided by Chiron. Recombinant CpPLD was produced as an endotoxin-free material, as described (7). Endotoxin levels were determined in all of the CpPLD preparations used in the study by the limulus amebocyte lysate assay (BioWhittaker Cambrex), and they were between 0.05 and 0.1 endotoxin unit per microgram of protein.

Carotid Artery T-Cell Lines from Atherosclerotic Patients. Upon the approval of the local Ethical Committee, carotid plaques were obtained by endoarterectomy from seven patients (four males and three females, mean age 65; range 58–70 y) with atherosclerotic arteriopathy. Patients (*Cp-pos*) were selected on the basis of detectable serum levels of anti-*C. pneumoniae* antibodies (Eurospital). Fresh plaque-derived T cells (1×10^5) were stimulated by immobilized anti-CD3 (OKT3) monoclonal antibody (5 μg/mL) in round-bottom microwell plates (30). After 72 h of stimulation, supernatants were collected and assayed for IL-17 and IL-21 by ELISA (R&D Systems and eBioscience, respectively). At day 15, T-cell blasts of each *Cp-pos* plaque T-cell line were stimulated with CpPLD (10 μg/mL), outer membrane protein (OMP)-2 (10 μg/mL), TT (0.5 μg/mL), or PPD (10 μg/mL) in the presence of autologous APCs for 48 h in ELISPOT microplates coated with anti–IL-17 antibody (eBioscience). The antigen-specific T-cell response was also evaluated in five patients without *anti-C. pneumoniae* antibody (*Cp-neg*), used as control. At the end of culture period, the number of IL-17 SFCs were counted as described (34).

Generation of T-Cell Clones from Atherosclerotic Plaques. Plaque-derived T cells were cloned under limiting dilution, and clones were screened for responsiveness to *C. pneumoniae* sonicated EB (10^4 inclusion forming units per mL), CpPLD (10 μg/mL), or *H. pylori* lysate (10 μg/mL) as described (8).

Assessment of T-Cell Clones Cytokine Profile. To assess their cytokine production, T-cell blasts (10^6 cells per mL) of each clone were stimulated for 36 h with phorbol-12-myristate 13-acetate (PMA; 10 ng/mL) in wells coated with anti-CD3 mAb, as reported (35). At the end of culture period, duplicate samples of each supernatant were assayed for IFN-γ, IL-4 (BioSource International), IL-17 (R&D Systems), and IL-21 (eBioscience).

Assay for T-Cell Clone Helper Function for Monocyte TF and MMP9 Production. T-cell blasts of *C. pneumoniae*-specific clones (8×10^5/mL) were cocultured for 16 h with autologous monocytes (4×10^5/mL) in the presence of medium or antigen CpPLD (10 μg/mL). At the end of the culture period, TF was quantitated as reported (18). To assess their ability to induce MMP-9 activity by monocytes, PLD-specific T-cell clones were cocultured with autologous monocytes in the presence of medium or CpPLD antigen (10 μg/mL). The MMP-9 activity was measured by using the SensoLyte MMP-9 ELISA Kit (Anaspec) according to the manufacturer's instructions.

Preparation of Monocytes and HUVEC. Human monocytes and HUVECs were prepared as described (36, 37). Monocytes were cultured in RPMI 1640 10% FCS in the presence of CpPLD (5μg/mL) or PBS, as control.

Real-Time PCR Analysis. Total RNA was isolated by using TRIzol solution (Invitrogen) according to the manufacturer's instructions. RNA was reverse-transcribed and amplified, using Light Cycler (Roche), with the appropriate primers for GAPDH, IL23p19, IL12p40, IL6, IL1β, CCL-20, CCL4, CCL2, CXCL9, VCAM, and ICAM, as described (34).

Detection of Cytokines and Chemokines in Culture Supernatants. Culture supernatants were collected, and the amount of IL-23, IL-6, IL-1β, CCL-4,

CCL-2, CCL-20, CXCL-9, CCL-20, VCAM-1, and ICAM-1 was quantified by ELISA (eBioscience, R&D Systems, and RayBiotech) according to the manufacturer's instructions. The inhibition of ^{3}H-thymidine incorporation into DNA of CCL64 mink lung epithelial cells was used as a sensitive bioassay for TGF-β (38).

ACKNOWLEDGMENTS. We thank the Italian Ministry of University and Research, the Italian Ministry of Health, the University of Florence, the Istituto Superiore di Sanità, and the Fondazione Cassa di Risparmio di Padova e Rovigo for supporting our research.

1. Schmiel DH, Miller VL (1999) Bacterial phospholipases and pathogenesis. *Microbes Infect* 1:1103–1112.
2. Ghannoum MA (2000) Potential role of phospholipases in virulence and fungal pathogenesis. *Clin Microbiol Rev* 13:122–143.
3. Oude Weernink PA, López de Jesús M, Schmidt M (2007) Phospholipase D signaling: Orchestration by PIP2 and small GTPases. *Naunyn Schmiedebergs Arch Pharmacol* 374: 399–411.
4. Wolf K, Fischer E, Hackstadt T (2000) Ultrastructural analysis of developmental events in Chlamydia pneumoniae-infected cells. *Infect Immun* 68:2379–2385.
5. Peters J, et al. (2005) Silencing or permanent activation: Host-cell responses in models of persistent Chlamydia pneumoniae infection. *Cell Microbiol* 7:1099–1108.
6. Campbell LA, Kuo CC (2004) Chlamydia pneumoniae—an infectious risk factor for atherosclerosis? *Nat Rev Microbiol* 2:23–32.
7. Ciervo A, Mancini F, Cassone A (2007) Transcription, expression, localization and immunoreactivity of Chlamydophila pneumoniae Phospholipase D protein. *Microb Pathog* 43:96–105.
8. D'Elios MM, et al. (1997) Different cytokine profile and antigen-specificity repertoire in Helicobacter pylori-specific T cell clones from the antrum of chronic gastritis patients with or without peptic ulcer. *Eur J Immunol* 27:1751–1755.
9. Bäck M, Ketelhuth DF, Agewall S (2010) Matrix metalloproteinases in atherothrombosis. *Prog Cardiovasc Dis* 52:410–428.
10. Oppmann B, et al. (2000) Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* 13: 715–725.
11. Stockinger B, Veldhoen M (2007) Differentiation and function of Th17 T cells. *Curr Opin Immunol* 19:281–286.
12. Bettelli E, et al. (2006) Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 441:235–238.
13. Bettelli E, Oukka M, Kuchroo VK (2007) T(H)-17 cells in the circle of immunity and autoimmunity. *Nat Immunol* 8:345–350.
14. Lim HW, Lee J, Hillsamer P, Kim CH (2008) Human Th17 cells share major trafficking receptors with both polarized effector T cells and FOXP3+ regulatory T cells. *J Immunol* 180:122–129.
15. Mancini F, Savarino A, Losardo M, Cassone A, Ciervo A (2009) Characterization of the serological response to phospholipase D protein of Chlamydophila pneumoniae in patients with acute coronary syndromes. *Microbes Infect* 11:367–373.
16. Elhage R, et al. (2003) Reduced atherosclerosis in interleukin-18 deficient apolipoprotein E-knockout mice. *Cardiovasc Res* 59:234–240.
17. Lee TS, Yen HC, Pan CC, Chau LY (1999) The role of interleukin 12 in the development of atherosclerosis in ApoE-deficient mice. *Arterioscler Thromb Vasc Biol* 19:734–742.
18. Benagiano M, et al. (2003) T helper type 1 lymphocytes drive inflammation in human atherosclerotic lesions. *Prod Nat Acad Sci* 100:6598–6663.
19. Benagiano M, et al. (2005) Human 60-kDa heat shock protein is a target autoantigen of T cells derived from atherosclerotic plaques. *J Immunol* 174:6509–6517.
20. Whitman SC, Ravisankar P, Daugherty A (2002) IFN-γ deficiency exerts gender-specific effects on atherogenesis in apolipoprotein E-/- mice. *J Interferon Cytokine Res* 22: 661–670.
21. Park H, et al. (2005) A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. *Nat Immunol* 6:1133–1141.
22. Langrish CL, et al. (2005) IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J Exp Med* 201:233–240.
23. Korn T, et al. (2007) IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. *Nature* 448:484–487.
24. Zhu J, Yamane H, Paul WE (2010) Differentiation of effector CD4 T cell populations (*). *Annu Rev Immunol* 28:445–489.
25. Eid RE, et al. (2009) Interleukin-17 and interferon-gamma are produced concomitantly by human coronary artery-infiltrating T cells and act synergistically on vascular smooth muscle cells. *Circulation* 119:1424–1432.
26. Toschi V, et al. (1997) Tissue factor modulates the thrombogenicity of human atherosclerotic plaques. *Circulation* 95:594–599.
27. Falk E, Shah PK, Fuster V (1995) Coronary plaque disruption. *Circulation* 92:657–671.
28. Chen S, et al. (2010) IL-17A is proatherogenic in high-fat diet-induced and Chlamydia pneumoniae infection-accelerated atherosclerosis in mice. *J Immunol* 185:5619–5627.
29. Hoover DM, et al. (2002) The structure of human macrophage inflammatory protein-3α /CCL20. Linking antimicrobial and CC chemokine receptor-6-binding activities with human β-defensins. *J Biol Chem* 277:37647–37654.
30. Galkina E, Ley K (2009) Immune and inflammatory mechanisms of atherosclerosis (*). *Annu Rev Immunol* 27:165–197.
31. Acosta-Rodriguez EV, et al. (2007) Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat Immunol* 8:639–646.
32. Ghannam S, et al. (2011) CCL20 and β-defensin-2 induce arrest of human Th17 cells on inflamed endothelium in vitro under flow conditions. *J Immunol* 186:1411–1420.
33. Homey B, et al. (2000) Up-regulation of macrophage inflammatory protein-3 alpha/ CCL20 and CC chemokine receptor 6 in psoriasis. *J Immunol* 164:6621–6632.
34. Codolo G, et al. (2008) Borrelia burgdorferi NapA-driven Th17 cell inflammation in lyme arthritis. *Arthritis Rheum* 58:3609–3617.
35. Amedei A, et al. (2006) The neutrophil-activating protein of Helicobacter pylori promotes Th1 immune responses. *J Clin Invest* 116:1092–1101.
36. Del Prete G, et al. (1995) Th1 and Th2 T-helper cells exert opposite regulatory effects on procoagulant activity and tissue factor production by human monocytes. *Blood* 86: 250–257.
37. Tedesco F, et al. (1997) The cytolytically inactive terminal complement complex activates endothelial cells to express adhesion molecules and tissue factor procoagulant activity. *J Exp Med* 185:1619–1627.
38. Wolf G, Mueller E, Stahl RA, Ziyadeh FN (1993) Angiotensin II-induced hypertrophy of cultured murine proximal tubular cells is mediated by endogenous transforming growth factor-beta. *J Clin Invest* 92:1366–1372.

Supporting Information

Benagiano et al. 10.1073/pnas.1111833109

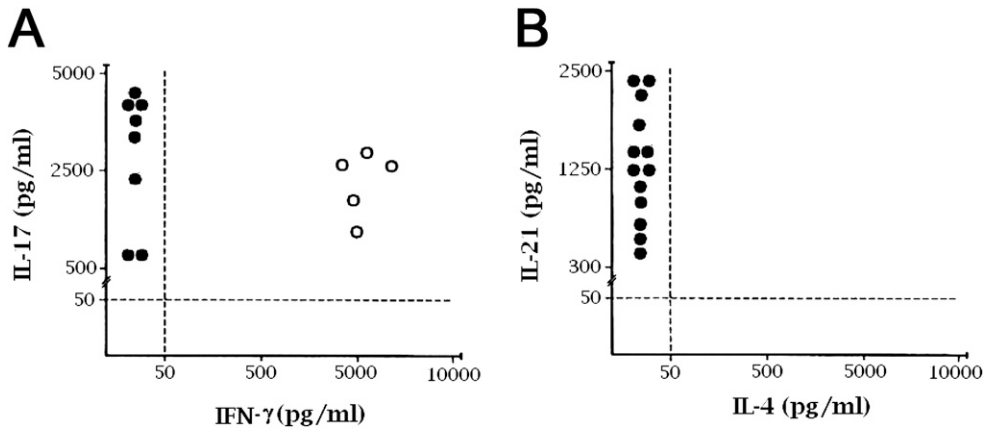

Fig. S1. *C. pneumoniae* antigen-induced cytokine production by plaque-infiltrating T-cell clones. *C. pneumoniae*-specific Th clones were stimulated with the appropriate antigen, and IL-17 and IFN-γ (*A*) and IL-21 and IL-4 (*B*) production was measured in culture supernatants. In unstimulated cultures, levels of IL-17, IFN-γ, IL-21, and IL-4 were consistently <20 pg/mL.

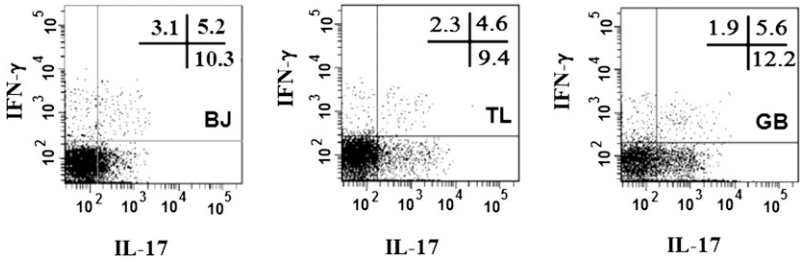

Fig. S2. IL-17 and IFN-γ intracellular cytokine staining of plaque-infiltrating T cells. Cells were stained for surface and intracellular markers with the following mAbs for flow cytometry: anti–CD4-PerCP, anti–IL-17-PE, and anti–IFN-γ-FITC (Becton Dickinson). Samples were stimulated with 25 ng/mL PMA plus 1 μg/mL ionomycin in the presence of brefeldin A (1 μg/mL). Dot plots expression of IL-17 and IFN-γ on $CD4^+$ T cells of three representative T-cell lines are shown.

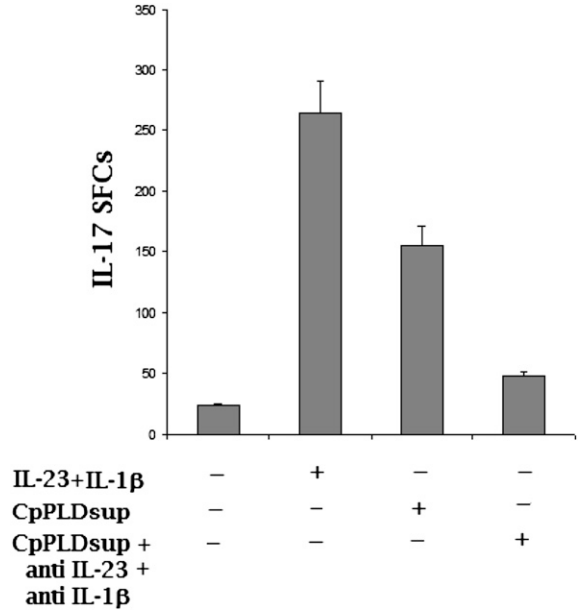

Fig. S3. Effect of CpPLD-cultured monocytes supernantants on Th17 cell differentiation. Enriched $CD4^+$ T cells were purified by using the StemSep Human T-cell enrichment kit (Voden Medical Instruments) were activated by anti-CD3 mAb (OKT3) with or without CpPLD-cultured monocytes supernatants (CpPLDsup); with IL-23 plus IL-1β; or with CpPLDsup plus blocking anti–IL-23 and anti–IL-1β. Recombinant human IL-2 was added to the cultures on days 4 and 7. After 10 d, cells were washed and restimulated for 48 h in enzyme-linked immunospot microplates coated with anti–IL-17 mAb. IL-17 spot-forming cells were then counted by using an automated reader. Values are the mean ± SD number of spot-forming cells (SFCs) per 10^5 cultured cells over background levels.

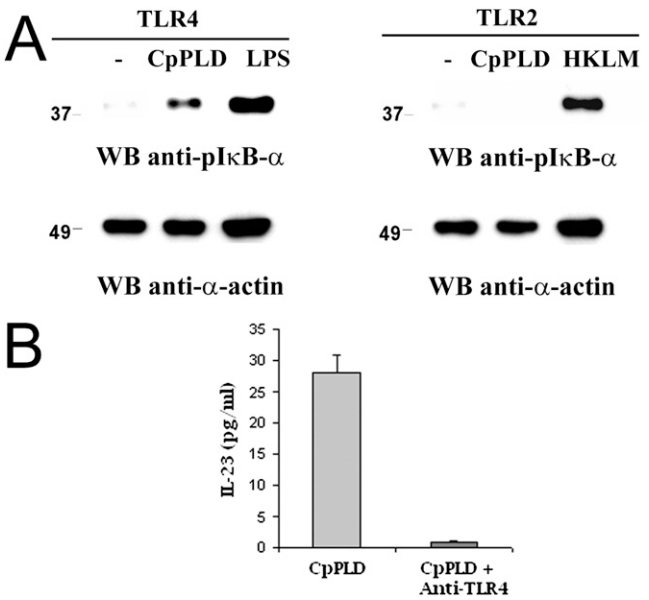

Fig. S4. Engagement of Toll-like receptor 4 (TLR-4) by CpPLD and its involvement in the production of interleukin-23 (IL-23). (*A*) HEK 293 cells expressing either TLR-2 or TLR-4 were incubated with CpPLD. As positive controls, *Listeria monocytogenes* was used for TLR-2–expressing cells, and lipopolysaccharide (LPS) was used for TLR-4–expressing cells. Mock cells represent the negative control. Cell lysates were subjected to SDS/PAGE and immunoblotted with anti–phospho-IκB-α antibodies. Total α-actin antibody was used as a control for equal loading. (*B*) Cells were either not preincubated or were preincubated for 2 h with 20 μg/mL of an anti–TLR-4 blocking antibody and then exposed to CpPLD. After 12-h culture, supernatants were collected and analyzed by ELISA for levels of the secreted IL-23. The bar on the left shows IL-23 secretion after CpPLD stimulation, and the bar on the right shows IL-23 secretion after a 2-h preincubation with 20 μg/mL of an anti–TLR-4 blocking antibody followed by stimulation with CpPLD. Values in *B* are the mean ± SD of four independent experiments by using triplicate samples.

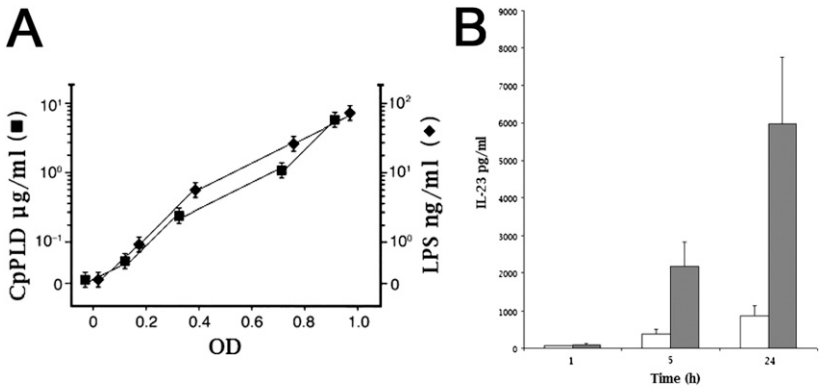

Fig. S5. Dose-dependent effect of CpPLD on TLR4 activation and timecourse of IL-23 monocyte production after CpPLD stimulation. (*A*) Activation of NF-κB in HEK 293 cells transfected with plasmid-encoding human TLR4. Parallel culture samples of hTLR4-transfected HEK 293 cells were stimulated with graded concentrations of CpPLD (squares) or LPS (diamonds). The NF-κB activation in each sample was quantified as OD values after 24 h of stimulation. Results of a representative experiment are reported. (*B*) Production of IL-23 after monocyte stimulation with CpPLD (gray bars) (10 μg/mL) or LPS (white bars) (1 μg/mL) at the indicated time points. Shown are representative results from one of four independent experiments.

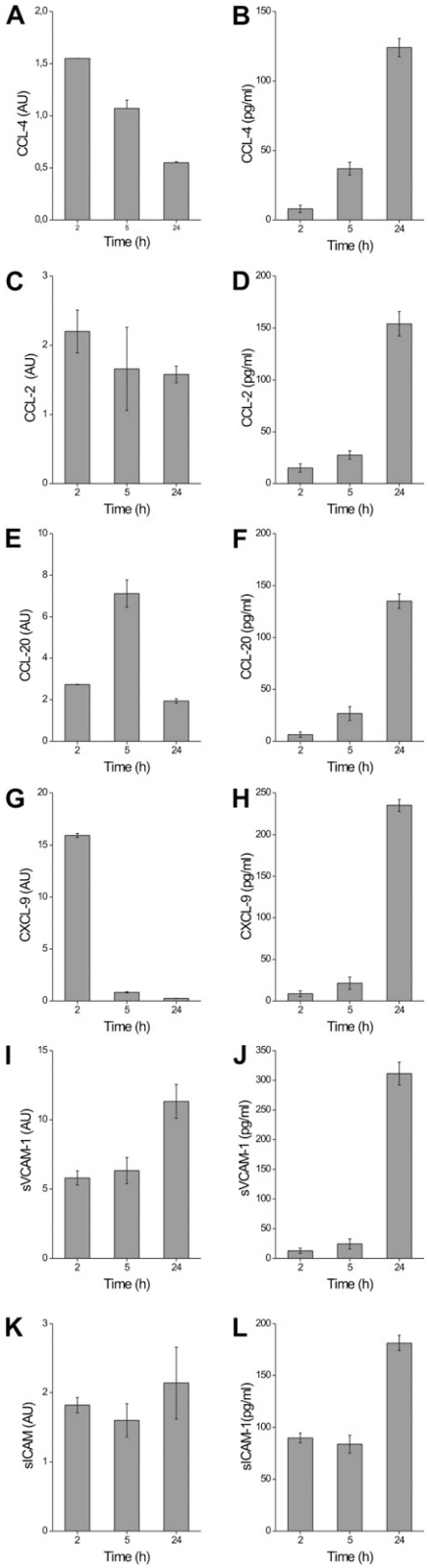

Fig. S6. Kinetics of the synthesis and production of CCL-4, CCL-2, CCL-20, CXCL-9, VCAM-1, and ICAM-1 by HUVEC stimulated with CpPLD. (*A, C, E, G, I,* and *M*) Levels of CCL-4, CCL-2, CCL-20, CXCL-9, VCAM-1, and ICAM-1 mRNAs were determined by quantitative real-time PCR at the indicated time points after CpPLD stimulation. AU, arbitrary units. (*B, D, F, H, L,* and *N*) Levels of CCL-4, CCL-2, CCL-20, CXCL-9, sVCAM-1, and sICAM-1 proteins were determined by ELISA at the indicated time points after CpPLD stimulation. Values are the mean ± SD of four independent experiments using triplicate samples.

Table S1. Antigen specificity of T-cell clones isolated from atherosclerotic plaques

Patients and source of T cells	Total no. of $CD4^+$ T-cell clones obtained	No. of clones reactive to: (%)	
		C. pneumoniae	*H. pylori*
C. pneumoniae positive patients			
AM	30	5 (16)	0
BJ	43	9 (21)	0
ST	28	4 (14)	0
HE	34	7 (20)	0
TL	27	6 (22)	0
GB	35	8 (23)	0
PF	26	5 (19)	0
C. pneumoniae negative patients			
FB	34	0	0
GM	41	0	0
BB	35	0	0
RG	29	0	0
AC	26	0	0

T-cell clones were cocultured with medium alone or *C. pneumoniae* sonicated elementary bodies (10^4 inclusion forming units per mL), or *H. pylori* lysate (10 μg/mL) in the presence of irradiated autologous APCs. Proliferative responses were measured after 3 d.

Table S2. Effect of addition in culture of anti-DR or anti-DQ monoclonal antibodies on the proliferative response to CpPLD by atherosclerotic plaque T-cell clones reactive to CpPLD

T-cell clones	Proliferative response in the presence of		
	Isotype control	Anti-DR	Anti-DQ
1. AM 3	83 ± 8	1.4 ± 0.2	82 ± 7
2. BJ 4	32 ± 5	0.8 ± 0.1	34 ± 4
3. ST 2	51 ± 9	1.2 ± 0.3	50 ± 6
4. HE 9	91 ± 6	1.4 ± 0.2	89 ± 5
5. TL 7	34 ± 7	1.5 ± 0.3	33 ± 4

T-cell clones were stimulated by CpPLD (10 μg/mL) in the presence of irradiated autologous APCs treated with anti–HLA-DR (clone G46-6) or anti–HLA-DQ (clone TU169) (5 μg/mL final concentration) mAbs to know the MHC restriction elements. Anti–HLA-DR resulted consistently in virtual abrogation of the proliferative response by T-cell clones to CpPLD, whereas anti–HLA-DQ was unable to affect CpPLD-induced T-cell clone proliferation. Proliferative response (MI) were measured after 3 d. Values are MI ± SD.