# C-Peptide Induces Chemotaxis of Human CD4-Positive Cells

## Involvement of Pertussis Toxin–Sensitive G-Proteins and Phosphoinositide 3-Kinase

**Daniel Walcher,[1] Milos Aleksic,[1] Verena Jerg,[1] Vinzenz Hombach,[1] Arthur Zieske,[2] Satoki Homma,[2] Jack Strong,[2] and Nikolaus Marx[1]**



**Increased levels of C-peptide, a cleavage product of proinsulin, circulate in patients with insulin resistance and early type 2 diabetes, a high-risk population for the development of a diffuse and extensive pattern of arteriosclerosis. The present study examined the effect of C-peptide on $CD4^+$ lymphocyte migration, an important process in early atherogenesis. C-peptide stimulated $CD4^+$ cell chemotaxis in a concentration-dependent manner. This process involves pertussis toxin–sensitive G-proteins as well as activation of phosphoinositide 3-kinase (PI 3-K). Biochemical analysis showed that C-peptide induced recruitment of PI 3-K to the cell membrane as well as PI 3-K activation in human $CD4^+$ cells. In addition, antidiabetic peroxisome proliferator–activated receptor γ–activating thiazolidinediones inhibited C-peptide–induced $CD4^+$ cell chemotaxis as well as PI 3-Kγ activation. Finally, immunofluorescence staining of thoracic artery specimen of diabetic patients showed intimal $CD4^+$ cells in areas with C-peptide deposition. Thus, C-peptide might deposit in the arterial intima in diabetic patients during early atherogenesis and subsequently attract $CD4^+$ cells to migrate into the vessel wall. *Diabetes* 53:1664–1670, 2004**

Patients with metabolic syndrome or type 2 diabetes exhibit an increased propensity for the development of a scattered and extensive pattern of arteriosclerosis. Typically, these insulin-resistant patients demonstrate increased serum levels of C-peptide, a cleavage product of proinsulin, released into the bloodstream in amounts equimolar to those of insulin (1). Despite several reports suggesting that C-peptide might modulate insulin or glucagon release from rat pancreas (2), it has been accepted for a long time that C-peptide possesses little or no biological activity. However, recent data suggest that C-peptide binds to specific yet unidentified cell surface receptors, thereby stimulating intracellular signaling processes (3), such as activation of Na-K-ATPase or stimulation of mitogen-activated protein kinase. These processes involve pertussis toxin–sensitive G-protein–coupled receptors as well as activation of protein kinase C and phosphoinositide 3-kinase (PI 3-K). Moreover, our group recently demonstrated significantly higher intimal C-peptide deposition in thoracic artery specimens from young diabetic subjects compared with matched nondiabetic control subjects as determined by immunohistochemical staining. C-peptide colocalized with monocytes/macrophages in the arterial intima of artery specimen from diabetic subjects and induced monocyte chemotaxis in vitro (4). We examined the effect of C-peptide on another type of vascular cells, $CD4^+$ T-cells, the major T-cell population in arteriosclerosis, and hypothesized that C-peptide may activate T-cell chemotaxis, thus potentially promoting T-cell migration to sites of developing lesions in diabetic patients.

Recruitment of T-cells into developing lesions is a critical step in early atherogenesis. This stage is characterized by an increased endothelial permeability with intimal deposition of plasma compounds such as LDL or C-reactive protein (5), as well as by an activation of the endothelium itself. In addition, $CD4^+$ cells are attracted during this phase by chemotactic proteins such as RANTES and enter the vessel wall as naïve TH0-cells. In the subendothelium, these cells differentiate to TH1-cells, releasing proinflammatory mediators such as tumor necrosis factor-α and γ-interferon, which then activate other cells in the vasculature (6). Hitherto, the role of C-peptide on $CD4^+$ cell migration is unexplored.

Thiazolidinediones (TZDs), such as rosiglitazone and pioglitazone, are a group of novel antidiabetic agents that are clinically used to treat patients with type 2 diabetes (7). On a molecular level, TZDs activate the nuclear transcription factor peroxisome proliferator–activated receptor-γ (PPAR-γ), thus regulating the expression of various target genes. In addition to their metabolic action, these agents exhibit direct, mainly anti-inflammatory effects on vascular cells (8). In human $CD4^+$ cells, PPAR-γ–activating TZDs have been shown to modulate proliferation (9,10) as well as TH1-cytokine expression

From the [1]Department of Internal Medicine II–Cardiology, University of Ulm, Ulm, Germany; and [2]Louisiana State University, Health Sciences Center, New Orleans, Louisiana.

Address correspondence and reprint requests to Nikolaus Marx, MD, Department of Internal Medicine II–Cardiology, University of Ulm, Robert-Koch-Strasse 8, D-89081 Ulm, Germany. E-mail: nikolaus.marx@medizin.uni-ulm.de.

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D.W. and M.A. contributed equally to this work.

PI 3-K, phosphoinositide 3-kinase; PPAR, peroxisome proliferator–activated receptor; TZD, thiazolidinedione.

(11), but nothing is known about the effect of TZDs on $CD4^+$ cell migration.

Given the importance of $CD4^+$ cell recruitment in early atherogenesis, we examined the chemotactic effect of C-peptide in human $CD4^+$ cells and analyzed intracellular signaling pathways involved. In addition, we investigated whether PPAR-γ–activating TZDs might modulate C-peptide–induced $CD4^+$ cell migration.

## RESEARCH DESIGN AND METHODS

**Human $CD4^+$ cell chemotaxis assay.** Human $CD4^+$ cells were isolated from freshly drawn blood of healthy volunteers using gradient centrifugation with subsequent magnetic bead isolation, as previously described (12). After isolation, $CD4^+$ cells were cultured in serum-free media for 16 h. T-cell chemotaxis was assayed under serum-free conditions in a 48-well microchemotaxis chamber (Neuroprobe). Wells in the upper and lower chambers were separated by a polyvinylpyrrolidone-free polycarbonate membrane (pore size 5 μm; Costar). $CD4^+$ cells at a density of $5 \times 10^5$/ml were incubated for 2.5 h with recombinant C-peptide or RANTES (Sigma) before migrated cells on the bottom face of the filter were stained and counted under the light microscope. Cells were counted in five random high-power fields per well. Checkerboard analysis was performed to differentiate chemotactic from chemokinetic activity. In some experiments, $CD4^+$ cells were treated with pertussis toxin, wortmannin, or LY294002 (both PI 3-K inhibitors) or pretreated for 15 min with PPAR-γ–activating TZDs (rosiglitazone or pioglitazone).

**Preparation of human T-cell membranes.** Human T-cell membranes were prepared by incubating cells in a lysis buffer that contained 10 mmol/l HEPES (pH 7.5), 3 mmol/l $MgCl_2$, 40 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 2 μg/ml pepstatin A, and 2 μg/ml aprotinin. After sonication, lysates were centrifuged at 15,000*g* for 10 min. The pellet was resuspended in radioimmunoprecipitation assay buffer (50 mmol/l Tris [pH 8.0], 150 mmol/l NaCl, 0.1% SDS, 0.5% DOC, 1% Nonidet P-40, and 1% Triton-X) and snap-frozen at −70°C.

**Immunoblotting.** Aliquots of the membrane fraction were boiled in Laemmli buffer before running on SDS-PAGE. Immunoblotting was performed by running the samples on SDS-PAGE and then electrotransferring onto nitrocellulose membranes (Amersham Pharmacia Biotech, Amersham, England), blocking with 5% skim milk in TBS buffer with 0.1% Tween 20 for 1 h, and incubating with 1:200 of the primary antibody (goat anti-human PI 3-Kγ [Santa Cruz] or rabbit anti-human PI 3-Kγ; rabbit anti-human p85 [Upstate]) and 1:10,000 dilution of the secondary antibody (anti-goat or anti-rabbit horseradish peroxidase [DAKO]). Development was done by using enhanced chemiluminescence reagents (Pearce, Rockford, IL) according to the manufacturer's specifications.

**Phosphatidylinositol kinase assay.** After isolation, human T-cells were incubated for 16 h in RPMI medium without serum. Cells that were pretreated with or without wortmannin, pertussis toxin, or TZDs were stimulated with 10 nmol/l C-peptide, 10 nmol/l heat-inactivated C-peptide, or 100 pg/ml RANTES. Standard PI 3-K activity assays were performed (4) using goat anti-human PI 3-Kγ (Santa Cruz) or rabbit anti-human PI 3-Kγ (Santa Cruz) antibodies.

**Immunofluorescence staining.** To examine the deposition of C-peptide in early arteriosclerotic lesions, we used postmortem artery specimen received from the multicenter cooperative project Pathobiological Determinants of Atherosclerosis in Youth, which collected material from 15- to 34-year-old trauma victims who were autopsied in forensic laboratories (13). Serial Cryostat sections of thoracic artery specimen were cut, air dried onto microscopical slides, and fixed in acetone at −20°C for 5 min. Staining for C-peptide was performed with a rabbit anti-human C-peptide antibody (Linco-Research). Human $CD4^+$ cells were identified by staining with a mouse anti-human CD4 antibody (Dako). Negative controls used type- and class-matched IgG at similar concentrations. Sections were preincubated with PBS that contained 5% respective serum. Primary antibodies (1:50, anti–C-peptide; 1:20, anti-CD4) diluted in PBS that contained 3% serum were added for 1 h at room temperature. After washing with PBS, Alexa Fluor 488-coupled (Molecular Probes) and carboxymethylindocyanine 3-Cy3-coupled (Dianova) goat anti-mouse and anti-rabbit IgG were added as secondary antibodies (dilution 1:1,000) for 45 min. Images were recorded with a confocal laser-scanning microscope (Leica).

**Statistical analysis.** Results of the experimental studies are reported as mean ± SD. Differences were analyzed by one-way ANOVA followed by the appropriate post hoc test. $P < 0.05$ was regarded as significant.

## RESULTS

**C-peptide induces migration of human $CD4^+$ cells in vitro.** To examine whether C-peptide induces $CD4^+$ cell migration, we subjected human $CD4^+$ cells to an in vitro chemotaxis assay using a modified Boyden chamber. Stimulation of $CD4^+$ cells with C-peptide induced cell migration in a concentration-dependent manner with a maximal induction of 2.1 ± 0.5 at 10 nmol/l ($P < 0.05$ vs. unstimulated cells; $n = 7$). Heat inactivation of C-peptide (10 nmol/l) abolished its migratory effect ($P < 0.05$; $n = 5$), excluding endotoxin contamination as the responsible mechanism (Fig. 1*A*). The extent of C-peptide–induced $CD4^+$ cell migration was similar to the effect of an established T-cell chemokine, RANTES, which led to a 2.1 ± 0.6-fold increase ($P < 0.05$; $n = 6$) in cell migration. It is interesting that combined stimulation of $CD4^+$ cells with C-peptide and RANTES did not have an additive migratory effect (Fig. 1*B*).

To specify the true nature of C-peptide's chemotactic activity, we conducted a checkerboard analysis using serial dilutions of C-peptide above and below the filter. As shown in Fig. 1*C*, $CD4^+$ cell migration depended on the presence of a C-peptide gradient between the upper and lower face of the filter, suggesting that C-peptide induces $CD4^+$ cell chemotaxis rather than chemokinesis.

**TZDs inhibit C-peptide–induced migration of human $CD4^+$ cells.** To examine the effect of antidiabetic PPAR-γ–activating TZDs on $CD4^+$ cell migration, we pretreated cells for 15 min with different concentrations of rosiglitazone or pioglitazone before performing stimulation with C-peptide. Both rosiglitazone and pioglitazone reduced C-peptide–induced $CD4^+$ cell chemotaxis in a concentration-dependent manner, with a maximal reduction to baseline levels at 5 μmol/l (Fig. 2*A*). None of the TZDs affected cell viability, as determined by trypan blue staining (data not shown).

To examine whether the effects of rosiglitazone and pioglitazone on C-peptide–induced T-cell migration are PPAR-γ–mediated, we performed similar experiments with the established non-TZD PPAR-γ activator GW1929. Pretreatment of human $CD4^+$ cells with GW1929 at 10 μmol/l abolished C-peptide–induced cell migration (Fig. 2*B*), suggesting that the TZD effects observed are PPAR-γ dependent.

**C-peptide–induced $CD4^+$ cell migration involves pertussis toxin–sensitive G-proteins and PI 3-K.** To investigate further intracellular signaling pathways involved in C-peptide–induced $CD4^+$ cell chemotaxis, we performed inhibition migration experiments. Treatment of human $CD4^+$ cells with pertussis toxin inhibited C-peptide–induced $CD4^+$ cell migration in a concentration-dependent manner, with a maximal reduction to baseline levels at 10 nmol/l pertussis toxin (Fig. 3*A*), suggesting that pertussis toxin–sensitive G-proteins are involved. Because some G-protein–coupled receptors activate PI 3-K and given the involvement of PI 3-K on T-cell motility (14–16), we next examined the role of PI 3-K. Treatment of $CD4^+$ cells with wortmannin or LY294002, two PI 3-K inhibitors, completely inhibited C-peptide–induced $CD4^+$ cell chemotaxis (Fig. 3*B*). Pertussis toxin, wortmannin, or LY294002 did not affect cell viability as examined by trypan blue staining (data not shown). These data suggest that C-peptide–

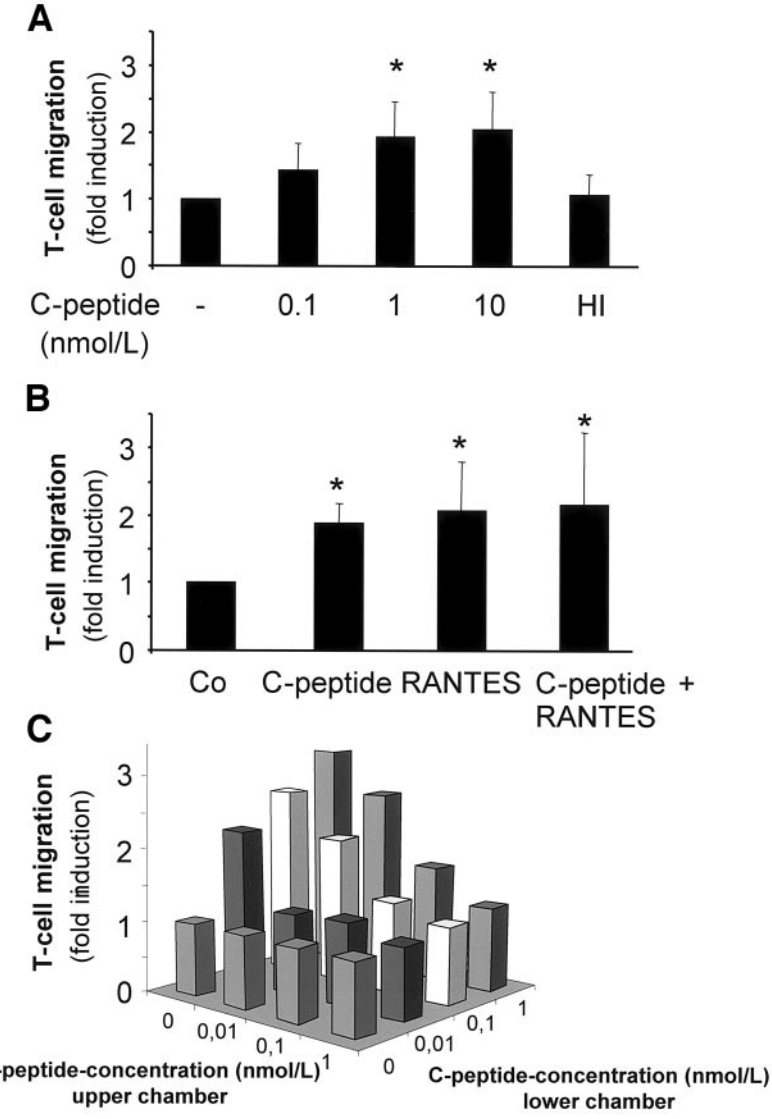


**FIG. 1. C-peptide induces CD4+ cell chemotaxis in vitro. *A*: Freshly isolated human CD4+ cells were incubated for 2.5 h with C-peptide at the concentrations indicated to assess the effect on cell migration in a modified Boyden chamber. Heat-inactivated C-peptide (HI) was used as a control. Data are expressed as fold induction of unstimulated cells. Bars represent mean ± SD ($n = 7$); $P < 0.05$ vs. unstimulated cells. *B*: The extent of C-peptide–induced (10 nmol/l) CD4+ cell migration is similar to the effect of the established T-cell chemokine RANTES (100 pg/ml). Also shown is the combined effect of C-peptide and RANTES on CD4+ cell migration. Data are expressed as fold induction of unstimulated cells. Bars represent mean ± SD ($n = 6$); $P < 0.05$ vs. unstimulated cells. *C*: Checkerboard analysis revealed that CD4+ cell migration depended on the presence of a C-peptide gradient between the upper and the lower face of the filter, suggesting that C-peptide induces CD4+ cell chemotaxis rather than chemokinesis. Data are expressed as fold induction of unstimulated cells. Bars represent the mean of three independent experiments.**

induced CD4+ cell migration involves pertussis toxin–sensitive G-proteins as well as PI 3-K.

**C-peptide activates PI 3-K in human CD4+ cells.** To gain further insight into C-peptide–induced PI 3-K activation, we examined the recruitment of PI 3-Kγ into the cell membrane. Incubation of CD4+ cells with C-peptide resulted in an association of PI 3-Kγ with CD4+ cell membranes. Two different anti–PI 3-Kγ antibodies showed similar results. Heat-inactivated C-peptide had no such effect. Consistent with former data on RANTES-induced recruitment of PI 3-K to the membrane, pretreatment of CD4+ cells with wortmannin did not inhibit C-peptide–mediated association of PI 3-Kγ with cell membranes (Fig. 4*A*). Given the involvement of class IA PI 3-Ks in insulin signaling (17), we investigated the role of this class of PI 3-Ks in C-peptide–induced cell activation. Western blot analysis of activated cells did not show an association of the regulatory subunit p85 with CD4+ cell membranes (data not shown), suggesting that C-peptide–induced PI 3-K activation involves only the p110γ isoform of PI 3-Ks.

Next, we examined the ability of PI 3-Kγ to generate phospholipids after C-peptide stimulation. C-peptide increased PI 3-Kγ activity in human CD4+ cells, whereas heat inactivation of C-peptide as well as pretreatment of cells with wortmannin abolished this effect. It is interesting that pretreatment of CD4+ cells with pertussis toxin also inhibited C-peptide–induced PI 3-Kγ activation, suggesting the involvement of pertussis toxin–sensitive proteins upstream of PI 3-K (Fig. 4*B*).

Because antidiabetic TZDs limited C-peptide–induced CD4+ cell chemotaxis, we investigated the effect of rosiglitazone and pioglitazone on C-peptide–mediated PI 3-K activation. Pretreatment (15 min) of human CD4+ cells with rosiglitazone or pioglitazone inhibited C-peptide's stimulatory effect on PI 3-K activity (Fig. 4*C*).

**C-peptide deposition and infiltration of CD4+ cells in early atherosclerotic lesions of diabetic subjects.** Given the importance of CD4+ cell recruitment in early atherogenesis and C-peptide's chemotactic effect on CD4+ cells shown here, we examined C-peptide deposition and CD4+ cell infiltration in early arteriosclerotic lesions of diabetic subjects. We used 21 postmortem thoracic aorta specimens from the Pathobiological Determinants of Atherosclerosis in Youth study in which subjects with $HbA_{1c}$ levels ≥8% were classified as diabetic (13) and compared them with 21 age-, sex-, and risk factor–matched nondia-

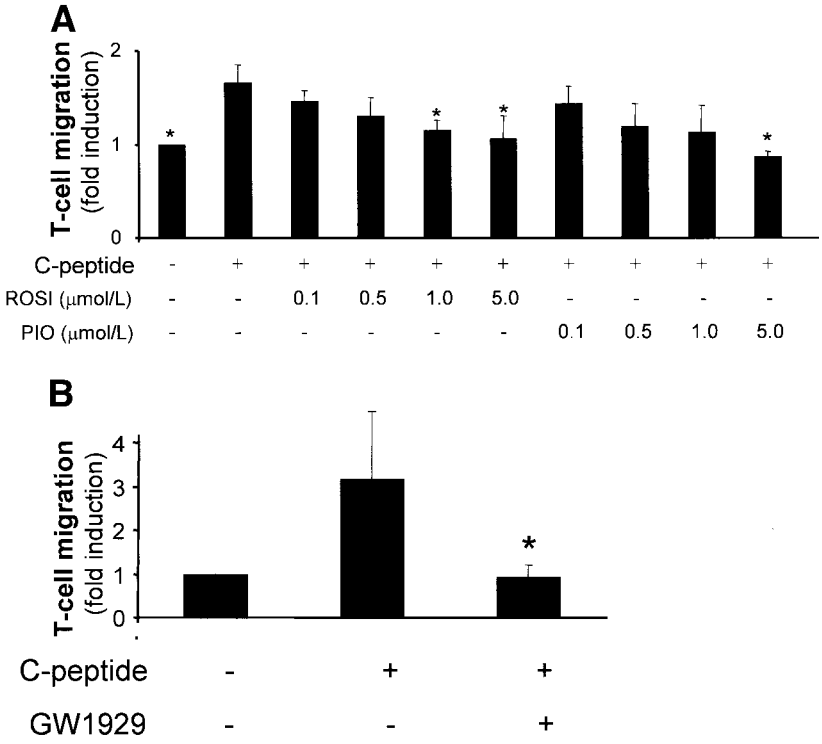


**FIG. 2. *A*: Antidiabetic TZDs inhibit C-peptide–induced CD4$^+$ cell migration. Freshly isolated human CD4$^+$ cells were pretreated with rosiglitazone (ROSI) or pioglitazone (PIO) for 15 min at concentrations indicated before migration experiments were performed. Data are expressed as fold induction of unstimulated cells. Bars represent mean ± SD ($n$ = 6); $P$ < 0.05 vs. unstimulated cells. *B*: GW1929, a non-TZD PPAR-γ activator, inhibits C-peptide–induced CD4$^+$ cell migration. Freshly isolated CD4$^+$ cells were pretreated with GW1929 (10 μmol/l) before migration experiments were performed. Data are expressed as fold induction of unstimulated cells. Bars represent mean ± SD ($n$ = 4); $P$ < 0.05 vs. unstimulated cells.**

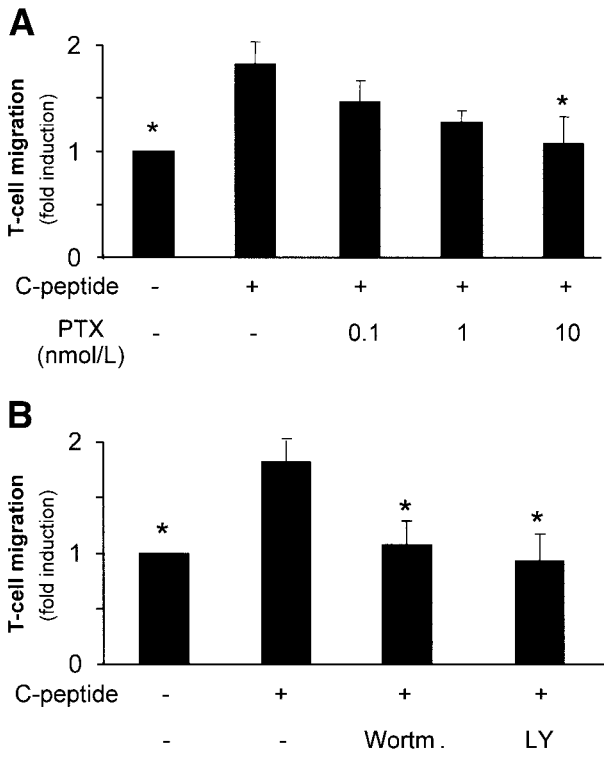


**FIG. 3. C-peptide–induced CD4$^+$ cell migration involves pertussis toxin–sensitive G-proteins as well as PI 3-K. *A*: Freshly isolated human CD4$^+$ cells were incubated with pertussis toxin (PTX) at concentrations indicated during migration experiments. Data are expressed as fold induction of unstimulated cells. Bars represent mean ± SD ($n$ = 3). *B*: Freshly isolated human CD4$^+$ cells were incubated with wortmannin (50 nmol/l) or LY294002 (500 nmol/l), two PI 3-K inhibitors, during migration experiments. Data are expressed as fold induction of unstimulated cells. Bars represent mean ± SD ($n$ = 4).**

betic control subjects. Recently, we showed that the extent of C-peptide deposition in these diabetic subjects exceeds that of nondiabetic control individuals (4). Immunofluorescence staining of artery sections from both groups revealed that intimal CD4$^+$ cells were detectable in 12 of 21 (57%) diabetic patients, but only in 2 of the 21 (9.5%) nondiabetic subjects. In these 12 diabetic subjects, immunofluorescence staining demonstrated intimal CD4$^+$ cells in areas with C-peptide deposition (Fig. 5*A–D* and *F*). Staining of parallel sections with isomatched IgGs at similar concentrations showed no immunoreactivity (Fig. 5*E* and *G*), thus affirming the specificity of the detected signals.

## DISCUSSION

The present study demonstrates that C-peptide, a cleavage product of proinsulin, exhibits chemotactic activity on human CD4$^+$ cells by involving pertussis toxin–sensitive G-proteins and PI 3-K activation. In addition, the study reports intimal CD4$^+$ cells in areas with C-peptide deposition in early atherosclerotic lesions of diabetic subjects as well as inhibition of C-peptide–induced CD4$^+$ cell migration by antidiabetic TZDs. These data raise for the novel hypothesis that C-peptide may promote early atherogenesis in patients with type 2 diabetes by initiating or promoting CD4$^+$ cell migration into developing lesions.

The effect of C-peptide on CD4$^+$ cell migration is similar to the chemotactic action of the well-established T-cell chemokine RANTES. It is interesting that C-peptide and RANTES had no additive effect on CD4$^+$ cell chemotaxis, suggesting that both agents share similar signaling pathways in these cells. Previous work has shown that C-peptide binds to a cell surface receptor, but this receptor remains unidentified (3). Work in renal cells implicates a pertussis toxin–sensitive G-protein–coupled receptor in mediating C-peptide's effects (18), and our data showing

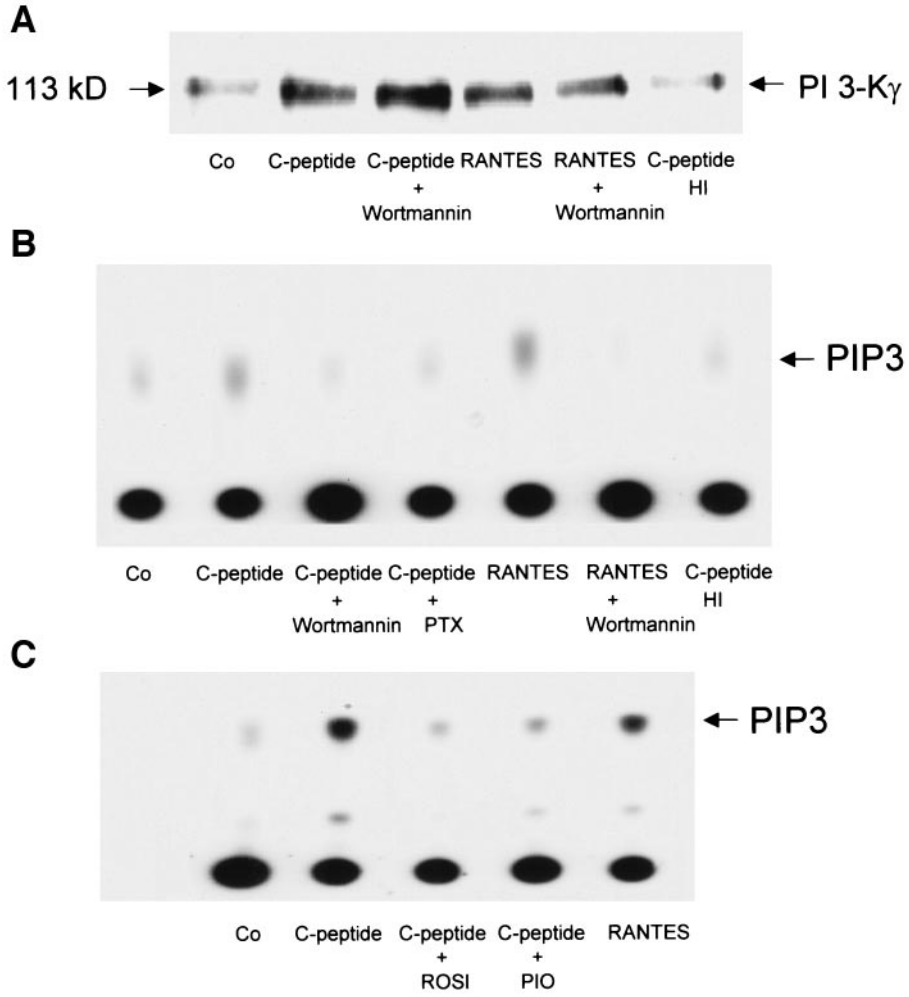


**FIG. 4. C-peptide activates PI 3-K in human CD4+ cells. *A*: C-peptide stimulation results in the translocation of PI 3-Kγ to the cell membrane in human CD4+ cells. Cells were stimulated with C-peptide (10 nmol/l) or RANTES (100 pg/ml) for 5 min before Western blot analysis of PI 3-Kγ on CD4+ cell membranes was performed. Similar results were obtained in three independent experiments. *B*: C-peptide activates PI 3-K. Human CD4+ cells were treated with C-peptide (10 nmol/l) or RANTES (100 pg/ml) for 5 min before PI 3-K activity assays were performed. Also shown are cells treated with C-peptide in the presence of wortmannin (50 nmol/l) or pertussis toxin (PTX) as well as CD4+ cells that were treated with HI C-peptide. Specific dots are labeled with an arrow (phosphatidylinositol-triphosphate). Three independent experiments yielded similar results. *C*: TZDs inhibit C-peptide–induced PI 3-K activation. Human CD4+ cells were pretreated with rosiglitazone (ROSI) and pioglitazone (PIO) for 15 min before cells were stimulated with C-peptide (10 nmol/l). After 5 min, PI 3-K activity assay was performed. Specific dots are labeled with an arrow ($PIP_3$).**

an inhibitory effect of pertussis toxin on C-peptide–induced CD4+ cell migration suggest that similar pathways are involved here. Downstream of these pertussis toxin–sensitive G-proteins, C-peptide stimulation results in translocation of PI 3-Kγ to the cell membrane as well as an increase in PI 3-Kγ activity. These results are in accordance with data in Swiss 3T3 fibroblasts (19), where C-peptide has been shown to activate PI 3-K. Previous work has reported that insulin signaling involves class IA PI 3-Ks (17), but our data, demonstrating a lack of an association of p85 with cell membranes, suggest that C-peptide–induced activation of CD4+ cells involves only PI 3-Kγ (the class IB enzyme) but not class IA PI 3-Ks.

Two TZDs, antidiabetic drugs used to treat patients with type 2 diabetes, completely inhibited C-peptide's chemotactic effect. Previous work (9,11) has shown that TZDs modulate proliferation as well as TH1-cytokine expression of human CD4+ cells. Our study extends the knowledge about TZDs' effects in these cells by demonstrating an inhibition of T-cell migration. TZDs are activators of the nuclear transcription factor PPAR-γ, thus regulating the expression of various target genes. It is interesting that a 15-min pretreatment of CD4+ cells with TZDs already prevented the rapid effect of C-peptide on PI 3-K activation, raising the question of a nontranscriptional effect of these agents in this context. Recent work by Chawla et al. (20) found that some effects of TZDs could occur independent of the presence of PPAR-γ, at least in cells of the monocytic lineage, and such mechanisms could potentially explain the rapid inhibition of PI 3-K activation observed here. However, our data showing similar inhibitory action on CD4+ cell migration by the non-TZD PPAR-γ activator GW1929 suggest that the effects of TZDs are PPAR-γ mediated. Further work should focus on the molecular mechanism of the effects of TZDs on PI 3-K activation.

The present study suggests an active role of C-peptide in the development of atherosclerotic lesions in patients with type 2 diabetes by showing intimal CD4+ cells in areas with C-peptide deposition in early atherosclerotic lesions

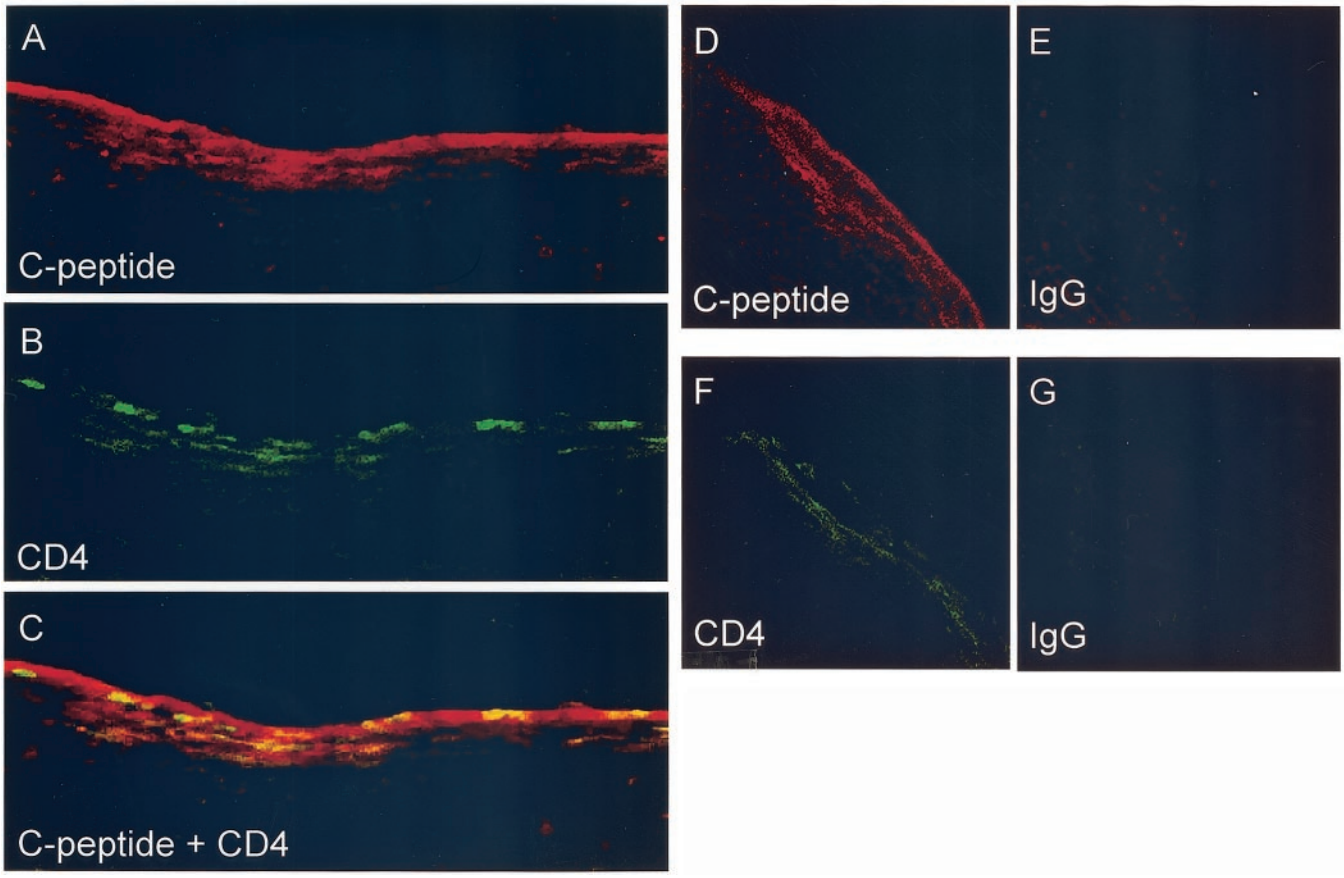


**FIG. 5. C-peptide deposition and intimal CD4$^+$ cell infiltration in thoracic aortic specimens from diabetic subjects. *A*: Immunofluorescence staining of C-peptide deposition in the intima (stained in red). *B*: CD4$^+$ cells in the same section stained in green. *C*: The yellow fluorescence (red and green) indicates colocalization of C-peptide with CD4$^+$ cells. Specimens from 12 diabetic subjects yielded similar results. *D* and *F*: C-peptide deposition and CD4$^+$ cell infiltration in a section from a different diabetic subject. *E* and *G*: Adjacent sections stained with similar concentrations of type- and class-matched IgG show no immunoreactive C-peptide (*E*) or CD4$^+$ cells (*G*), establishing the specificity of staining in *D* and *F*.**

of diabetic subjects. During endothelial dysfunction with increased endothelial permeability, C-peptide might deposit in the arterial intima in diabetic patients and subsequently attract CD4$^+$ cells to migrate into the vessel wall. Still, future studies should examine C-peptide deposition and CD4$^+$ cell infiltration in arteriosclerotic lesions from type 1 diabetic patients to link our observational study to possible mechanisms of disease.

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