CD40-CD40 Ligand Interactions in the Production of IL-12 and IFN-γ by Tuberculous Pleural Mononuclear Cells

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ABSTRACT

Background: Our previous study showed that purified protein derivative (PPD)-stimulated pleural mononuclear cells (PMC) from tuberculous pleurisy (Tbp) produced significantly more IFN-γ (10- to 70-fold) after *in vitro* PPD stimulation than freshly isolated pleural cells from malignant pleurisy. The present study was designed to determine whether blocking the CD40-CD40 ligand (CD40L) interaction decreases IFN-γ production by altering IL-12 levels. **Methods:** IL-12 and IFN-γ production after neutralizing anti-CD40L antibody treatment was compared to the efficacy of anti-CD80, anti-CD86, and a combination of anti-CD80 and CD86 (CD80+86) monoclonal antibodies (mAb). These activities were measured by enzyme-linked immunosorbent assays (ELISAs) and reverse transcription-polymerase chain reaction (RT-PCR), after *in vitro* stimulation with PPD antigen (Ag). **Results:** Neutralization of CD80, CD86 and CD80+86 did not decrease IFN-γ and IL-12 production in Tbp-PMC, whereas neutralization of CD40L significantly depressed IL-12 p40 and IFN-γ. In addition, neutralization of CD40L completely inhibited IL-12 p40 and IFN-γ mRNA expression. **Conclusion:** The CD40-CD40L interaction might play a major role in IL-12 and IFN-γ production in Tbp-PMC, thus contributing to protective immunity in human tuberculosis. **(Immune Network 2002;2(3):142-149)**

Key Words: CD40 ligand, tuberculous pleurisy, pleural mononuclear cells, CD80, CD86, PPD antigen, 30-kDa antigen

Introduction

Tuberculous pleurisy (Tbp) is a good model for understanding local cellular immunity. Tuberculous pleural effusions typically show a predominance of T lymphocytes (1,2), especially CD4+ T cells (2,3), and release high levels of interferon (IFN)-γ (4-7). IFN-γ, a product of activated T and NK cells, has a major role in activating cell-mediated immunity and contributes to a host's defense against intracellular bacterial infections (8,9). Purified protein derivatives (PPD), a mixture of low-molecular weight proteins from *Mycobacterium tuberculosis*, stimulate the production of cytokines implicated in Th1 differentiation, such as IFN-γ (10,11). In Tbp, PPD-stimulated pleural fluid cells produce higher amounts of IFN-γ than peripheral blood mononuclear cells (12). This local immune response, orchestrated by T cells and by a cytokine network, seems to be involved in the favorable outcome of Tbp patients, and may represent the protective immune response invoked to control the spread of *M. tuberculosis* infection (13).

Our previous study showed that interleukin (IL)-12 levels were significantly increased in pleural effusions from Tbp patients (14), and there is evidence that IL-12 is a potent stimulator of IFN-γ production (15). IL-12 is an inducible, heterodimeric, disulfide-linked cytokine composed of 35- and 40-kDa subunits encoded by separate genes (16). This cytokine induces IFN-γ production, primarily by NK cells, thereby activating the phagocytic cell system as a first line of natural defense. In addition, the early induction of IL-12 is necessary for the optimal generation

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of T helper type 1 (Th1) CD4+ cells (15). IL-12 mRNA production is elevated at the site of tuberculosis infection and neutralizing IL-12 suppressed the proliferative responses of pleural fluid cells to *M. tuberculosis*, suggesting that IL-12 plays a role in the human immune response to infectious agents *in vivo* (11).

CD40/CD40 ligand (CD40L) co-stimulation is an important regulator of Th1 responses (17). This co-stimulation has a potent effect on the production of IL-12 and IFN-γ in cells stimulated with T-cell-specific Ags (17). In addition, CD40L and IFN-γ synergistically restore IL-12 production in HIV-infected patients (18). However, the role of CD40/CD40L stimulation in tuberculosis is controversial. A recent study reported that the CD40L-mediated pathway of T-cell activation is dispensable for resistance to *M. tuberculosis* infection (19). This was in contrast to the enhanced susceptibility of patients with a defective CD40L gene (20) to other experimental infections with intracellular pathogens, including *Leishmania* (21).

The current study was undertaken to further characterize the Th1 regulatory mechanisms attributable to Th1 elevation in Tbp after *in vitro* stimulation with PPD Ag via CD40/CD40L interaction. The production of IFN-γ and IL-12 in PPD Ag-stimulated PMC from 12 patients with tuberculous pleurisy (Tbp-PMC) was compared with that of PMC from 10 patients with malignant effusions (MG-PMC). In response to Ag, both IL-12 and IFN-γ were found to increase significantly in Tbp-PMC. In addition, blocking CD40/CD40L interactions also decreased IL-12 and IFN-γ production in Tbp-PMC. Neither separate nor simultaneous neutralization of CD80 and CD86 led to a change in IL-12 and IFN-γ production.

Materials and Methods

Subjects. Twelve HIV-negative patients with Tbp, and without a history of previous anti-tuberculous therapy or diabetes mellitus were included in this study. Tbp was diagnosed by the histopathological finding of granulomas with caseous necrosis in pleural biopsies, or by a positive culture of *M. tuberculosis* from biopsy specimens. Pleural exudate cells were obtained from drained pleural fluid obtained routinely for diagnosis and as part of the treatment of the Tbp patients. Pleural effusions were also collected from 10 patients with newly diagnosed lung cancer with malignant effusion (MG).

Five healthy tuberculin reactors (HTR) exhibited skin reactions of more than 15 mm after an intradermal test with 5 units of PPD-RT23 (Statens Seruminstitut, Copenhagen, Denmark), drawn within 1 to 3 years of their PPD skin test examination, and had no previous history of clinical TB. Each of these healthy controls had received *Mycobacterium bovis* Bacille Calmette-Guerin (BCG) vaccinations as children. All patients and healthy volunteers consented to take part in this study.

Antigen and antibodies. Tuberculin PPD for *in vitro* assays was purchased from the Statens Seruminstitut (Copenhagen, Denmark), and was used at a final concentration of 1.0 mg/ml. Endotoxin content was measured by the *Limulus* amoebocyte lysate assay and was found to be below 1.5 pg/ml in PPD Ag. Neutralizing anti-human CD40L antibodies (5c8) were kindly provided by Dr. Shigeaki Nonoyama (Tokyo Medical and Dental University, Tokyo, Japan). Neutralizing anti-human CD80 and CD86 were purchased from R&D Systems (Minneapolis, MN, USA). For flow cytometric analysis, fluorescein isothiocyanate (FITC)-labelled anti-CD3, and phycoerythrin (PE)-labelled anti-CD40L were purchased from PharMingen (San Diego, CA, USA).

Two-color immunocytometry. Fresh or mycobacterial antigen stimulated mononuclear cells were resuspended at 2×10^6 to 5×10^6 cells/ml in staining buffer (phosphate-buffered saline-2% human serum-1% bovine serum albumin-0.02% sodium azide) containing a saturated amount of antibodies, and incubated for 30 min at 4°C. After staining, the cells were washed three times with staining buffer and analyzed with a FACScalibur (Becton & Dickinson Immunocytometry, San Jose, CA, USA). Data were represented as two-dimensional contour maps. To obtain the percentage of a subpopulation, total counts were integrated in selected areas of contour plots. Background staining of the negative control Ig was subtracted from experimental values.

Preparation and stimulation of peripheral blood mononuclear cells. Venous blood was drawn from subjects into sterile blood-collection tubes and peripheral blood mononuclear cells (PBMC) were isolated by density sedimentation over Histopaque-1077 (Sigma, St. Louis, MO, USA). PBMC were then suspended at a density of 1×10^6 viable cells/ml in complete medium [RPMI 1640, (Gibco-BRL, Gaithersburg, MD, USA) with 10% fetal bovine serum (Gibco- BRL), sodium pyruvate, non-essential amino acids, penicillin G (100 IU/ml), and streptomycin (100μg/ml)]. Cells were then stimulated with PPD antigen (1.0μg/ml), phytohemagglutinin (PHA, 10μg/ml; Sigma), or lipopolysaccharide (LPS, 0.1μg/ml; Sigma) and incubated at 37°C in a 5% CO_2 humidified air atmosphere until being used for either RNA isolation or supernatant collection.

As a positive control in the evaluation of CD40L expression, PBMC were prepared by Ficoll-Hypaque

gradient centrifugation, and cultured with phorbol 12-myristate 13-acetate (10 ng/mL) (Sigma) and ionomycin (400 ng/mL) (Calbiochem-Novabiochem Corp, La Jolla, CA) for 8 h.

Enzyme-linked immunosorbent assay for IFN-γ, IL-12 p40, and IL-12 p70. Supernatants were collected from cultures of PBMC stimulated with PPD Ag at 18 (for IL-12 p40 and p70), and 96 (for IFN-γ) h, and were then frozen at 80C. The frozen supernatants were thawed at room temperature, and cytokine levels were measured with commercial assay kits for IFN-γ, IL-12 p70, and IL-12 p40 (PharMingen), according to the manufacturers' instructions. Cytokine concentrations in the samples were calculated with standard curves generated from recombinant cytokines, and the results were expressed in picograms per milliliter. The difference between duplicate wells was consistently less than 100% of the mean.

Reverse transcriptase polymerase chain reaction (RT-PCR). PMC and PBMC were collected and washed, and total RNA was isolated using an RNAgent kit (Promega, Madison, WI) from cultures at various time points. First-strand cDNA synthesis and PCR were performed as previously described (22). All the PCR reactions were performed using 1μl cDNA and 2 U AmpliTaq DNA polymerase (Perkin-Elmer) in 20-μl reaction mixtures in a thermocycler (Biometra Inc., Tampa, FL). Each cycle consisted of 1 min at 95°C, 1 min at 60°C, 1 min at 72°C, and a final extension step of 5 min at 72°C. An external control, the housekeeping gene β-actin, was used to normalize the starting amount of cDNA for each sample prior to PCR.

Statistical methods. The results are presented as the mean±SD. Statistical significance was calculated using Student's *t*-test, ANOVA or linear regression analysis.

Results

IFN-γ production in patients with tuberculous pleurisy, malignancy, and healthy tuberculin reactors. PMC was isolated from patients, cultured in the presence of PPD Ag for 96 h, and assayed by ELISA for IFN-γ production. To measure IFN-γ production in a logarithmic range, we performed IFN-γ ELISA with serially (20- to 100-fold) diluted supernatants. As shown in Fig. 1, the mean pleural fluid IFN-γ level was significantly higher in Tbp patients than in both patients with malignancy (2837.7±4620.6 versus 41.7 ±33.9 pg/ml, $p<0.001$), and HTR serum (8.1±7.8 pg/ml, $p<0.001$). In addition, the mean IFN-γ concentration in the supernatants from PPD-stimulated Tbp-PMC was significantly higher than the corresponding values for MG-PMC (48.0±18.8 versus 0.2 ±0.2 ng/ml; $p<0.001$), and HTR-PBMC (898.3± 402.5 pg/ml, $p<0.001$). IFN-γ levels were more than 16-fold higher in supernatants from Ag-stimulated Tbp-PMC than in those from freshly isolated Tbp-PMC, whereas MG-PMC exhibited smaller (3.7-fold) increases in IFN-γ production.

IL-12 p40 production in patients with tuberculous pleurisy, malignancy, and healthy tuberculin reactors. The levels of IL-12 p70 and p40 after co-culture with PPD for 18 h were determined using ELISA. In preliminary experiments, IL-12 p70 release correlated strongly with IL-12 p40 release (n=11, $r=0.81$, $p<0.001$; data not shown). Unstimulated Tbp-PMC showed detectable levels of IL-12 p70 (62.1±35.0 pg/ml), and these were significantly higher in Tbp patients than in patients with malignancy ($p<0.05$; data not shown).

As shown in Fig. 2(A), the mean IL-12 p40 production increased significantly in Tbp-PMC after stimulation with PPD when compared with cells before stimulation (1584.2±791.3 pg/ml versus 708.3±289.2 pg/ml, $p<0.01$), or when compared with the IL-12 p40 level in MG-PMC after stimulation with PPD (248.1±446.3 pg/ml, $p<0.001$). In addition, the mean IL-12 p40 concentrations in PPD-stimulated Tbp-PMC cultures were significantly higher than those in HTR-PBMC cultures (1584.2±791.3 versus 487.0±308.0 pg/ml, $p<0.001$). There was no significant difference in IL-12 p40 production between PPD-stimulated MG-PMC and HTR-PBMC (248.1±446.3 pg/ml versus 487.0±308.0 pg/ml, $p>0.05$).

We also observed that the production of IL-12 p40 in Tbp-PMC from individual patients correlated

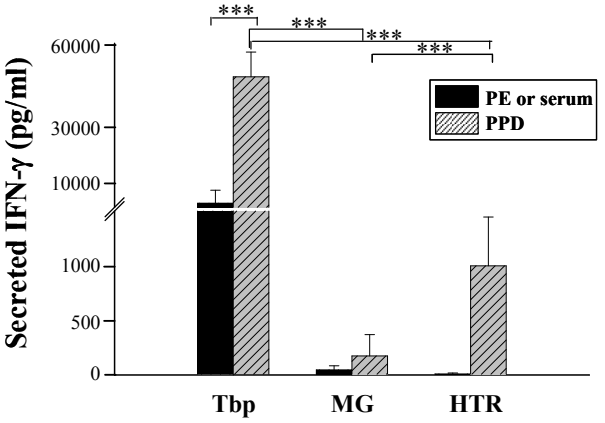

Figure 1. IFN-γ production of pleural mononuclear cells from the patients with tuberculous pleurisy after *in vitro* stimulation with the PPD antigen of *Mycobacterium tuberculosis*. Supernatants were prepared after the incubation periods indicated (96 h), and IFN-γ production was measured by ELISA. Values are means± SD of triplicate supernatant samples.

significantly with IFN-γ secretion 96 h after stimu-

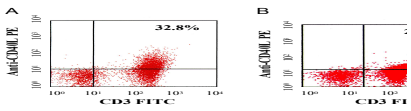

Figure 3. Phenotypic analysis by FACS of CD40L expression from Tuberc
and PBMC were cultured with 1μg/ml of PPD antigen from *M. tuberculo*
CD40L molecules in T cells by flow cytometry. Freshly isolated PMC
data correspond representative experiments. (A) PPD-stimulated PMC in
healthy tuberculin reactors. (C) PBMC after PMA plus ionomycin for 1 (

Figure 4. RT-PCR analysis of cDNA after *in vitro* stimulation with the PI
HTR-PBMC were cultured for various times, as indicated, in the presence of th
or
a PCR with primers specific for each cytokine gene was performed. The PC
Panel A shows IFN-γ mRNA expression in Tbp-PMC, *panel B* shows IL-12 p
IFN-γ and IL-12 p40 mRNA expression in HTR-PBMC. *M*, molecular weigh
RPMI (media control); *2*, mRNA from PPD-stimulated PMC/PBMC; *3*, mR
mRNA from PPD-stimulated PMC/PBMC with anti-CD86; *5*, mRNA from
and anti-CD86; *6*, mRNA from PPD-stimulated PMC/PBMC with anti-CD4
αB7-2

Figure 2. IL-12 p40 production of pleural mononuclear cells from the *Panel A* shows
the PPD antigen of *Mycobacterium tuberculosis*. Supernatants were prepared
production was measured by ELISA. Values are means±SD of triplicate
Tbp and malignant patients, and PBMC from HTR after an 18-h stimulation. *B*, Linear regression analysis of IL-12 p40 and IFN-γ
production in tuberculous pleurisy patients. IL-12 p40 and IFN-γ re
16 and 96 h. Significant correlation was found between IL-12 p40

Figure 5. IFN-γ and IL-12 p40 production by pleural mononuclear cells from
costimulatory molecules. IL-12 p40 (18 h) and IFN-γ (96 h) production wa
and IFN-γ production in Tbp-PMC induced by PPD Ag. means±SD of triplicate supernatant samples. *A*, IFN-γ production; and *B*, I

lation with PPD (n=11, r=0.7, p=0.01; Fig. 2B).

CD40 ligand expression in patients with tuberculous pleurisy and healthy tuberculin reactors. As a preliminary investigation into the CD40/CD40L interaction in PMC and PBMC, CD3 and CD40L expressing T cells were assessed by flow cytometry. The mean percentage of CD3+ T cells of HTR controls (n=5) increased from 45.2±6.5% to 60.9±24.2% in response to the PPD antigen. Similarly, CD3+ T cells in Tbp-PMC (n=7) increased from 78.0±2.5% to 90.5±12.5% in response to PPD.

One of the representative experiments for CD40L expression in response to mycobacterial antigen is shown in Fig. 3. The mean levels of CD40L/CD3 expression were after stimulation with PPD, increasing from 7.6±2.0% to 26.0±9.2%, and from 16.0± 5.7% to 34.3±7.4%, in HTR and Tbp-PMC, respectively. This indicates that CD40L expressing T cells of HTR-PBMC and Tbp-PMC preferentially respond to the PPD antigen of *M. tuberculosis*.

Effect of the neutralization of CD80, CD86, and CD40 ligands on PPD-induced IL-12 p40 and IFN-γ production in patients with tuberculous pleurisy and healthy tuberculin reactors.

RT-PCR: We assessed the effect of CD80, CD86, and CD40L neutralization on PPD-induced IL-12 p40 and IFN-γ mRNA expression. HTR-PBMC and Tbp-PMC were cultured in complete RPMI with or without PPD (1μg/ml) in the presence or absence of neutralizing antibodies to CD80, CD86, and CD40L (1μg/ml).

Total RNA was extracted at 6 and 96 h, and levels of cytokine mRNA were determined by RT-PCR. A representative experiment illustrating the pattern of IL-12 p40 and IFN-γ mRNA induced by the PPD in Tbp-PMC and HTR-PBMC is shown in Fig. 4. Cytokine mRNA production was evaluated by visual comparison of the intensity of the band representing PCR product from antigen-stimulated cells with those from freshly isolated (negative control) or PHA-stimulated cells (positive control). After PPD treatment, IFN-γ mRNA from PBMC was detected at 6 h, with increased detection at 48 h. IFN-γ mRNA levels then fell slightly at 96 h. IFN-γ mRNA expression was also clearly observed in PPD-stimulated Tbp-PMC in the presence of anti-CD80 or CD86 at 48 h. However, IFN-γ mRNA expression was low in PPD-stimulated Tbp-PMC with both anti-CD80 and CD86. Interestingly, IFN-γ mRNA expression was very low after neutralization of CD40L molecules at 48 h, and expression could not be observed at 96 h (Fig. 4A). In addition, IFN-γ mRNA levels were significantly reduced in PPD-stimulated PMC with anti-CD80, anti-CD86 or both at 96 h.

As shown in Fig. 4(B), IL-12 p40 mRNA expression after a 6-h stimulation in Tbp-PMC was similar to IFN-γ expression after 96 h. IL-12 p40 mRNA was clearly expressed in PPD-stimulated cells treated with anti-CD80, CD86, or CD80/86 antibodies after a 6-h stimulation. However, IL-12 p40 mRNA was not detected after neutralization of CD40L molecules in PPD-stimulated cells. In HTR-PBMC, IFN-γ mRNA expression was more increased compared with that of Tbp-PMC, whereas IL-12 p40 mRNA

expression was similar or slightly lower to that of Tbp-PMC (Fig. 4C).

ELISA: As shown in Fig. 5(A), PPD-induced IL-12 p40 levels were equivalent to levels in cultures containing neutralizing anti-CD80, CD86, or CD80/86 antibodies ($p > 0.01$). PPD-induced IL-12 p40 levels in the presence of anti-CD80 were relatively higher than those observed after PPD stimulation. However, co-culture with a neutralizing antibody to CD40L led to a significant decrease in IL-12 p40 production in Tbp-PMC (1.7-fold, $p < 0.05$; Fig. 5A). In addition, IFN-γ production in Tbp-PMC was significantly reduced by co-culture with the neutralizing antibody to CD40L (2-fold, $p < 0.05$; Fig. 5B). IFN-γ levels stimulated with PPD after co-culture with neutralizing antibodies to CD80, CD86, or both were similar to levels detected after PPD only. In HTR-PBMC, IL-12 p40 and IFN-γ levels were approximately equivalent to those of Tbp-PMC.

Discussion

An effective immune response against *M. tuberculosis* requires the coordinated interaction of APC and lymphocytes, and therefore secondary signals, such as the interaction of co-stimulatory molecules, are very important for T cell activation. In this study, we determined the effect of CD80, CD86 and CD40L co-stimulatory molecule neutralization on IFN-γ and IL-12 responses to PPD Ag in Tbp-PMC. PPD-stimulated Tbp-PMC was found to express significantly more ($p < 0.001$) IL-12 than Ag-stimulated MG-PMC, and IL-12 production in individual patients correlated with PPD Ag-induced IFN-γ secretion after stimulation for 96 h. In addition, blocking CD40/CD40L interactions decreased IL-12 and IFN-γ production in Tbp-PMC.

IL-12 plays a crucial role in IFN-γ induction by T lymphocytes (11,23,24), and IL-12-deficient mice are highly susceptible to infection by *M. tuberculosis* (25). Interactions between CD40 on antigen-presenting cells and CD40L on T cells enhance the Th1 response by inducing macrophages and dendritic cells (17,26,27) to produce interleukin-12 (IL-12), and by enhancing the expression of co-stimulatory molecules of the B7 family on antigen-presenting cells (28-30).

Interaction between the CD40 receptor on APC and its ligand (CD40L) on activated T cells plays a critical role in immunity to intracellular pathogens by up-regulating the production of IL-12 (31,32). CD40- or CD40L-deficient mice have an increased susceptibility to leishmanial infection, and show an impaired priming of Th1-type cells, correlating with a lack of activation of the macrophage effector functions required for parasite clearance (21,33). By contrast, CD40L-deficient mice show no difference in susceptibility to *M. tuberculosis* infection compared to wild-type mice (19), suggesting that cell-mediated immunity and protection against mycobacteria develop independently of CD40L.

The current study suggests that mycobacterial Ag-stimulated IL-12 and IFN-γ production by Tbp-PMC and HTR-PBMC involves CD40/CD40L interactions. Our evidence also partially agrees with others who showed that CD40L expression correlated directly with *M. tuberculosis*-stimulated IFN-γ production by PBMC from tuberculosis patients and HTR (34). This group also showed that a CD40L agonist increased *M. tuberculosis*-induced IFN-γ production by PBMC, and anti-CD40 or anti-CD40L antibodies reduced IFN-γ production. It is therefore possible that additional stimulation of the CD40/CD40L signaling pathway in mycobacterium-infected antigen presenting cells (APC) may further enhance IL-12 production, and consequently Th1 protective immunity.

Infection of dendritic cells with live *M. tuberculosis* bacilli results in increased APC surface expression of the co-stimulatory molecules CD54, CD40, and CD80, as well as MHC class I molecules (35). Additionally, another study showed that infection of a conditionally immortalized dendritic cell line with *M. tuberculosis* resulted in up-regulation of CD80 and CD86 co-stimulatory molecules, and the induction of several inflammatory cytokines, including tumor necrosis factor-alpha and IL-6, IL-1β, and IL-12 (36). However, in the present study, neutralization of CD80, CD86, or both did not affect IL-12 and IFN-γ production. One possible explanation for these differences might be that the antigen-expressing cells in Tbp-PMC used in our study might function differently from dendritic cells. We are now in the process of determining which cells are the principal APC in Tbp-PMC.

In terms of IFN-γ production, our data correlate well with other reports (11). Tuberculous pleural fluid lymphocytes underwent greater blastic transformation and produced more IFN-γ than pleural lymphocytes of tuberculin-positive non-tuberculous patients, whereas the opposite occurred in peripheral lymphocytes. In tuberculin-negative non-tuberculous patients, there were no lymphoblastic responses in either the pleural fluid or peripheral blood. Our data, taken together with these previous findings, suggest clonal expansion of mycobacterial Ag-responding T lymphocytes in the pleural compartment of Tbp patients. A recent report also indicated that IFN-γ may provide an afferent feedback signal that enhances IL-12 p70 expression through selective up-regulation of IL-12 p35 mRNA levels (37). Based on these results and our own data, we hypothesize that positive feedback via IL-12-IFN-γ

contributes to the characteristic cell-mediated immune response and facilitates the development of Th1 cells.

The results reported here suggest that CD40/CD40L interactions contribute to local protective immunity in Tbp patients via increased IL-12 and IFN-ɣ production. Future studies will clarify the molecular mechanisms of Th1 protective immunity via CD40/CD40L interactions.

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