OX40 Ligand Fusion Protein Delivered Simultaneously With the BCG Vaccine Provides Superior Protection Against Murine *Mycobacterium tuberculosis* Infection

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*Mycobacterium tuberculosis* infection claims approximately 2 million lives per year, and improved efficacy of the BCG vaccine remains a World Health Organization priority. Successful vaccination against *M. tuberculosis* requires the induction and maintenance of T cells. Targeting molecules that promote T-cell survival may therefore provide an alternative strategy to classic adjuvants. We show that the interaction between T-cell–expressed OX40 and OX40L on antigen-presenting cells is critical for effective immunity to BCG. However, because OX40L is lost rapidly from antigen-presenting cells following BCG vaccination, maintenance of OX40-expressing vaccine-activated T cells may not be optimal. Delivering an OX40L:Ig fusion protein simultaneously with BCG provided superior immunity to intravenous and aerosol *M. tuberculosis* challenge even 6 months after vaccination, an effect that depends on natural killer 1.1 (NK1.1) cells. Attenuated vaccines may therefore lack sufficient innate stimulation to maintain vaccine-specific T cells, which can be replaced by reagents binding inducible T-cell costimulators.

BCG, which is the current vaccine against *Mycobacterium tuberculosis* (*M. tuberculosis*), is effective against infantile tuberculosis meningitis and miliary tuberculosis [1] but fails to confer reliable protection against pulmonary tuberculosis in adults [2, 3]. Furthermore, tuberculosis drug treatment is lengthy and costly, and reduced compliance has led to the increase in multidrug-resistant strains [4, 5]. Because BCG confers significant protection in young children and provides cost-effective prevention from tuberculosis for developing countries, it would be desirable to modify the existing BCG vaccine to provide better protection. Indeed, many current vaccination strategies seek to improve immune priming to BCG through utilization of modified BCG strains or prime-boost strategies where an initial immune response to BCG is enhanced by a heterologous or homologous antigen boost [6, 7].

Protective immunity to *M. tuberculosis* requires CD4+ T cells that secrete type 1 cytokines. Although these cells are induced during natural infection, they do not ultimately limit the disease. The goal therefore is to design a vaccine that is superior to the pathogen in induction of CD4+ Th1 and CD8+ T cells. Efficient induction of acquired immunity relies on robust innate immune responses. Antigen-presenting cells play a role in priming antigen-specific T cells but must also provide sufficient stimulation to override activation-induced cell death. Similarly, cytokines produced by natural killer (NK) cells contribute to an environment conducive to CD4+ Th1 and CD8+ T-cell development and sustain later lung CD4+ T-cell effector/memory antymycobacterial responses [8–10]. Because
induction of cellular responses by vaccination in humans can be difficult, pathogen epitopes or proteins are often administered with innate immune-stimulating adjuvants. In 1989 Charles Janeway referred to these adjuvants as “the immunologists’ dirty little secret” [11] because their precise composition and mode of action are not known. The use of “adjuvants” that stimulate acquired immunity directly with greater precision is therefore appealing. In addition to T-cell receptor signaling, T cells require a succession of costimulatory signals to survive. The majority of costimulatory signals required later in the activation process are induced and so make interesting targets for use as a T-cell–specific adjuvant.

OX40 (CD134, ACT 35, Tnfrsf4) is an ideal target because it is predominantly expressed on recently activated T cells but absent on naive or resting memory T cells and is found on NK cells and NK T-cell populations required for antimycobacterial immunity [12]. On naive T cells the kinetics of OX40 expression is between 12 hours and 2–3 days, depending on the T-cell stimulus [13]. On memory T cells, however, reexpression of OX40 is faster (1–4 hours) [13]. Targeting OX40 therefore provides a strategy for boosting a small proportion of relevant T cells in the context of vaccination. OX40 can act independently of any other signal to promote survival or as a costimulator with T-cell receptor signaling to drive growth and clonal expansion [14]. Three copies of OX40 bind the trimeric ligand OX40L (Tnfsf4, gp34, CD252) [15], which is induced on a number of cell types, predominantly antigen-presenting cells [12]. The criteria for expression of OX40L on the variety of cell types that express it are less clear. OX40L on antigen-presenting cells and/or B cells appears 24 hours following CD40 or Toll-like receptor ligation. Interleukin 18 (IL-18) and thymic stromal lymphopoetin are also reported to increase OX40L expression [16, 17], and it is likely that various inflammatory cytokines impact on OX40L levels as described for tumor necrosis factor (TNF) on airway smooth muscle cells [18].

We hypothesized that ligation of OX40 during BCG-specific T-cell activation would provide superior protection upon *M. tuberculosis* challenge and provide a novel adjuvant that targets a relevant subset of acquired, rather than innate, immunity. We now show that a genetic deletion of OX40L that targets a relevant subset of acquired, rather than innate, immunity. We now show that a genetic deletion of OX40L that targets a relevant subset of acquired, rather than innate, immunity. We now show that a genetic deletion of OX40L that targets humoral immunity. We now show that a genetic deletion of OX40L that targets a relevant subset of acquired, rather than innate, immunity. We now show that a genetic deletion of OX40L that targets a relevant subset of acquired, rather than innate, immunity. We now show that a genetic deletion of OX40L that targets a relevant subset of acquired, rather than innate, immunity. We now show that a genetic deletion of OX40L that targets a relevant subset of acquired, rather than innate, immunity. We now show that a genetic deletion of OX40L that targets a relevant subset of acquired, rather than innate, immunity. We now show that a genetic deletion of OX40L that targets a relevant subset of acquired, rather than innate, immunity. We now show that a genetic deletion of OX40L that targets a relevant subset of acquired, rather than innate, immunity. We now show that a genetic deletion of OX40L that targets a relevant subset of acquired, rather than innate, immunity. We now show that a genetic deletion of OX40L that targets a relevant subset of acquired, rather than innate, immunity. We now show that a genetic deletion of OX40L that targets a relevant subset of acquired, rather than innate, immunity. We now show that a genetic deletion of OX40L that targets a relevant subset of acquired, rather than innate, immunity. We now show that a genetic deletion of OX40L that targets a relevant subset of acquired, rather than innate, immunity.

### Materials and Methods

#### Ethics Statement

This study was carried out in accordance with the recommendations in the Guide for the Use of Laboratory Animals of Imperial College London. All animal procedures and care conformed strictly to the UK Home Office Guidelines under the Animals (Scientific Procedures) Act 1986, and the protocols were approved by the Home Office of Great Britain (license no. 70/6646). All surgeries were performed under anesthesia, and all efforts were made to minimize suffering with a strict implementation of the replacement, reduction, and refinement principles.

#### Mice

Six- to eight-week-old female C57BL/6 mice were purchased from Harlan Olac Ltd. OX40L−/− mice, on a C57BL/6 background, were a gift from Kazuo Sagamura (Tohoku University School, Sendai, Japan). These mice do not harbor any gross abnormalities or apparent immunological defects when unchallenged (data not shown). All mice were kept in pathogen-free conditions according to Home Office guidelines, maintained in biosafety level 3 facilities, and given sterile water, mouse chow, and bedding.

#### BCG and *M. tuberculosis*

BCG Pasteur (Pasteur Institute, Paris, France) and *M. tuberculosis* H37Rv were grown in either Middlebrook 7H9 medium (Difco), supplemented with 10% albumin-dextrose-catalase (ADC; Difco), 0.2% glycerol, and 0.05% Tween 80 or on Middlebrook 7H11 agar plates supplemented with 10% oleic acid-albumin-dextrose-catalase (OADC; Difco), 0.2% glycerol, and 10 μg/mL amphotericin B. Bacteria for infection were obtained from late logarithmic growth phase cultures. The number of bacteria was assessed by measurement of optical density (OD) at 600 nm, where an OD of 1 represented 10⁸ colony-forming units (CFU)/mL.

#### Mouse Infections and Treatment

C57BL/6 and OX40L−/− mice were anesthetized with isoflurane and subcutaneously infected with 2 × 10⁷ CFU of BCG Pasteur (in 200 μL). In some experiments BCG-infected mice were challenged intravenously (5 × 10⁵ CFU/mL, in 200 μL) or via an aerosol route (50–100 bacilli per mouse) with *M. tuberculosis* H37Rv at different times after BCG infection, as noted in the text. Some groups of mice were injected intraperitoneally with 100 μg in (200 μL) OX40L-murine immunoglobulin (Ig) G1 fusion protein (OX40L:Ig) constructed as previously described [19] on day 0. NK cell depletion was via intraperitoneal administration of 100 μg anti-NK1.1 antibody (CD161; Clone: PK136 eBiosciences) at the time of BCG vaccination and every 3 days thereafter until day 12. Untreated groups received an analogous quantity of...
IgG1 (Caltag). Mice were sacrificed at various time points after BCG and *M. tuberculosis* infection by dislocation of the cervical vertebrae. The spleens and inguinal lymph nodes (ILNs) of BCG-infected mice and the spleens and lungs of *M. tuberculosis*-infected mice were removed under aseptic conditions. Tissue was disrupted to a single cell suspension by lysis of red blood cells with 10% OADC, 0.2% glycerol, and 10 µg/mL amphotericin B for CFU quantification. Red blood cells were lysed by suspending pellets in ACK buffer (0.15 M ammonium chloride, 1 M potassium hydrogen carbonate, and 0.01 mM ethylenediaminetetraacetic acid; pH 7.2). Cell viability was assessed by trypan blue exclusion and cells resuspended in Roswell Park Memorial Institute 1640 medium containing 10% fetal calf serum, 2 mM/mL L-glutamine, 50 µg/mL streptomycin, and 50 µg/mL penicillin, and 50 µg/mL amphotericin B at 10⁶ cells/mL.

**Flow Cytometric Analysis**

Cells were stained for surface markers and analyzed by flow cytometry. All antibodies were purchased from BD Pharmingen, except anti-OX40-FITC (fluorescein isothiocyanate) (Serotec UK). In brief, 1 × 10⁶ cells were stained using anti-CD4-Allophycocyanin (APC) and anti-CD8-PerCP. Cells were also stained with combinations of FITC-labeled antibodies specific for CD44, OX40, DX5, and PE-labeled anti-CD45RB or anti-CD45RB or anti-CD11b-PE, and anti-OX40L-biotin, followed by streptavidin-APC (Caltag). All antibodies were diluted in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA)/0.05% sodium azide (PBA). Cells were stained for 30 minutes on ice, washed with PBA, and spun for 5 minutes at 1200 rpm. After washing, cells were then fixed for 20 minutes at room temperature with 2% formaldehyde/PBS. Cells were then washed in PBA and data were acquired on a FACSCalibur, and 30,000 lymphocyte events were analyzed with CellQuest Pro software (BD Biosciences).

**Enzyme-Linked Immunosorbent Spot Assays**

Sterile filter plates (Millipore) were coated with anti-interferon (IFN)-γ antibodies (Pharmingen International) in 0.1M carbonate/bicarbonate buffer at pH 9.6 overnight before washing and blocking with complete medium. Then 5 × 10⁵ cells were added to wells with four 1:2 serial dilutions. After stimulation with purified protein derivative (PPD-RT46, Statens Serum Institut), cells were cultured for 48 hours. The cells were removed by washing and the site of cytokine production detected by biotin-labeled rat antimurine IFN-γ monoclonal antibody, using BCIP/NBT (Sigma-Aldrich) as an alkaline phosphatase substrate. Spot-forming colonies were counted using a dissecting microscope and wells without PPD subtracted as background.

**Proliferation Assays**

For the proliferation assays, 2 × 10⁵ cells were added to wells with four 1:2 serial dilutions. Cells were stimulated with 0.1–100 µg/mL of PPD and incubated for 24 hours. Cells were subsequently pulsed with 25 µL of tritiated thymidine (Amersham) and incubated overnight. Cells were harvested using a Beta plate counter (Wallac).

**Cytokine Release Assays**

For the cytokine release assays, 2 × 10⁵ cells were stimulated with 100 µg/well of PPD and incubated for 48 hours. Supernatants were removed and the concentrations of IFN-γ and TNF-α in supernatants were quantified using OptEIA kits (Pharimingen) according to manufacturer’s instructions.

**Statistical Analysis**

Statistical significance was calculated using an unpaired Mann–Whitney test and Prism software. All P values of <.05 and ≤.01 were considered significant and are referred to as such in the text.

**RESULTS**

**Loss of OX40L Limits Cellular Immunity to BCG**

Administration of BCG subcutaneously to C57BL/6 mice led to a rapid increase of OX40 expression on CD4⁺ and CD8⁺ T cells in the ILN and spleen (Figure 1A) that was maintained for prolonged periods. Consequently, the number of OX40⁺ T cells was also significantly elevated after BCG vaccination (Figure 1B). Conversely, OX40L on macrophages was only transiently expressed and low or absent on ILN and spleen dendritic cells (Figure 1C). To verify the importance of OX40L in the immune response to BCG, BCG was administered subcutaneously to wild-type C57BL/6 and OX40L⁻/⁻ mice. Naive OX40L⁻/⁻ mice have no gross abnormalities and comparable cell numbers to wild-type mice in the spleen and ILN (data not shown). However, a targeted deletion of OX40L resulted in strikingly reduced total cell numbers in the ILN (Figure 1D) and spleen (Figure 1E) compared with wild-type C57BL/6 controls at 14 days after BCG administration, owing to reduced T-cell numbers. Activation of splenic CD4⁺ (Figure 1F) and CD8⁺ (data not shown) T cells was also reduced. Thus it would appear that in the absence of OX40L, there is inefficient priming of the T-cell response to BCG.

**Ligation of OX40 In Vivo Enhances Th1 CD4⁺ and CD8⁺ Responses to BCG**

We next tested whether administration of an OX40L:lg fusion protein to wild-type mice acted as an adjuvant to the BCG. OX40L:lg was delivered intraperitoneally at the same time as BCG was delivered subcutaneously; the elicited immune response was evaluated at days 7, 14, and 21. CD4⁺ and CD8⁺
Coadministration of OX40L:IG With BCG Vaccine Augments Protection to Subsequent M. tuberculosis Challenge

To test the implications of enhanced T-cell responses and Th1-associated cytokines, vaccinated and control mice were challenged by intravenous injection of $5 \times 10^5$ virulent M. tuberculosis 42 days after initial vaccination. Fourteen days after M. tuberculosis challenge, soluble Th1 cytokine production by lung and splenic cells after 48 hours of incubation with PPD was elevated in M. tuberculosis-challenged mice previously vaccinated with BCG and OX40L compared with those vaccinated with BCG alone or mock vaccinated controls (data not shown). Enzyme-linked immunosorbent spot analysis of T cells from the same mice revealed them to be a dominant source of IFN-γ (Figure 3A and 3B) and TNF. Total number of cells in the lung (Figure 3C) and spleen (Figure 3D)
was also significantly raised by combining BCG with OX40L:Ig on day 14 after *M. tuberculosis* challenge compared with the control groups. The number of CFUs recovered after *M. tuberculosis* challenge from the lungs (Figure 3E) and spleen (Figure 3F) of BCG-vaccinated mice was reduced by approximately 1 log compared with mock-vaccinated controls at 14 days after challenge. A further 1-log reduction over and above that of BCG alone was observed in organs from mice that received BCG along with the OX40L:Ig adjuvant (Figure 3E and 3F).

To mimic the more natural route of infection with *M. tuberculosis*, we next investigated whether BCG administered with an OX40L:Ig adjuvant conferred greater protection against aerosolized *M. tuberculosis*. C57BL/6 mice sham treated or vaccinated with BCG alone or BCG and OX40L:Ig were challenged after 28 days with *M. tuberculosis* H37Rv via the aerosol route using a concentration of bacteria that provides approximately 50–100 bacteria per total lung. Protection was assessed 4 and 6 weeks later. We chose these later time points...
Figure 3. The role of OX40/OX40L in immunity to BCG and Mycobacterium tuberculosis (M. tuberculosis) challenge. Five mice per group were inoculated subcutaneously with phosphate-buffered saline (cont.), BCG plus control immunoglobulin (Ig) (BCG), or BCG + OX40L:Ig and challenged with $5 \times 10^5$ M. tuberculosis (strain R37rv) intravenously or 50–100 bacilli by aerosol. In mice intravenously challenged 42 days after vaccination, interferon (IFN)–γ spot-forming colonies (SFCs) from purified protein derivative–stimulated lung cells (A) or splenocytes (B) was enumerated by enzyme-linked immunosorbent spot assay (mean + SD of 5 mice per group) 14 days after challenge. In the same mice, total numbers of viable cells (C and D) and colony-forming units (CFUs) (E and F) in the lung (C and E) and spleen (D and F) 14 days after M. tuberculosis challenge were determined. In aerosol-challenged mice (G and H), the number of M. tuberculosis CFUs was determined by diluting homogenates of lung in mice challenged with M. tuberculosis 28 days (G) or 180 days (H) after BCG vaccination. All graphs show results for 4–5 mice per group and are representative of 2–3 independent experiments. *$P < .05$, **$P < .01$ by Mann–Whitney test (in H, significance is presented for BCG vs BCG + OX40L:Ig).
after challenge to show long-term protection using our adjuvanted strategy. As with intravenous challenge with *M. tuberculosis*, the number of CFUs recovered after *M. tuberculosis* challenge from the lungs (Figure 3G) and spleen (data not shown) of BCG-vaccinated mice was reduced by approximately 1 log compared with mock vaccinated controls at 4 and 6 weeks after challenge. A further 1-log reduction over and above that of BCG alone was observed in organs from mice that received BCG along with the OX40L:Ig adjuvant at both time points (Figure 3G). This further limitation of *M. tuberculosis* was also apparent when the interval between vaccination and challenge was extended to 180 days and CFUs were analyzed in an early time course up to the sampling points shown for the intravenous *M. tuberculosis* route (Figure 3H).

**Importance of NK1.1⁺ Cell Responses to Superior Protection in OX40L:Ig-Treated BCG-Vaccinated Mice**

Because OX40 is expressed on NK1.1⁺ cells and its ligation enhances the development of Th1 responses, we assessed their importance in augmented immunity to BCG vaccine and subsequent protection from *M. tuberculosis*. Administration of BCG subcutaneously to C57BL/6 mice led to enhanced NK cells (DX5⁺CD4⁺CD8⁻) in the spleen relative to saline control mice at days 7, 14, and 21 after vaccination (Figure 4A). Coadministration of intraperitoneal OX40L:Ig at time of vaccination resulted in an increase in NK cell numbers above that of BCG alone (Figure 4A). Administration of an anti-NK1.1—depleting antibody at time of vaccination, and every 3 days thereafter until day 12 after vaccination, abolished NK cells at days 7 and 14, but numbers were restored to baseline levels by day 21 (Figure 4A). The significance of the enhanced NK cell response in OX40L:Ig-treated mice to development of T-cell immunity was demonstrated by the enhanced spleen cell proliferation to PPD relative to saline or BCG-alone treated mice (Figure 4B). Furthermore, the enhanced protection achieved by combining BCG vaccination with OX40L:Ig on intravenous *M. tuberculosis* was abolished when NK1.1⁺ cells were depleted at time of vaccination (Figure 4C).

**DISCUSSION**

We show that in the absence of OX40L, immunity to BCG vaccination is severely impaired, unlike the dispensable role of another TNF superfamily member, LIGHT [20]. Furthermore, provision of an OX40 agonist at the time of BCG vaccination affords superior immunity and subsequently better control of *M. tuberculosis* compared with BCG vaccination alone. We are the first to demonstrate the adjuvant potential of OX40 agonists for mycobacterial disease, whereas efficacy is also documented for hepatitis B surface antigen [21], Simian immunodeficiency virus [22], murine cytomegalovirus [23], and, in conjunction with anti-cytotoxic T lymphocyte antigen (CTLA)–4, *Leishmania donovani* [24]. As in our study, OX40 agonists result in a Th1-cytokine–dominated response. OX40 ligation increases interleukin 2 (IL-2) production from effector T cells [13] and IL-2Rα, which increases IFN-γ in environments rich in interleukin 12 (IL-12) and IL-18 [25, 26]. OX40 agonists also increase IL-12R expression and prevent the expression of CTLA-4, FOXP3, and interleukin 10 (IL-10) [27–30].
Multiple studies have attempted to improve the existing BCG vaccine through the generation of recombinant BCG (rBCG) strains or coadministration of the parental BCG strain with immunological adjuvants (for reviews, see [31, 32] and references therein). The first recombinant vaccine with greater efficacy in animal models than the parental BCG strain overexpresses Ag85B of _M. tuberculosis_ and confers up to a 1-log reduction in _M. tuberculosis_ CFUs. In a phase 1 trial in humans, this vaccine enhanced antigen-specific T-cell proliferation and cytokine production. There has been a concerted effort to develop further recombinant BCG vaccines that overexpress members of the Ag85 complex either alone or associated with other _M. tuberculosis_ antigens or recombinant cytokines. However, only occasionally do they exhibit any improvement to parental BCG in conferring protection to _M. tuberculosis_. Other notable efforts to develop superior rBCG vaccines are overexpression of a pore-forming cytolysin, lysteriolysin, from _Listeria monocytogenes_; reintroduction of selected genes lost during attenuation of BCG [33]; overexpression of mycobacterial latency associated antigens [34–37]; and expression of functional cytokines alone or in combination with mycobacterial antigens [38, 39]. Again, efficacy beyond the conventional BCG in conferring protection to tuberculosis is either unknown or limited. Innate immune molecules have been targeted as adjuvants to BCG vaccine. For example, IL-12, TNF, lactoferrin, and oligodeoxynucleotides containing CpG motifs enhance Th1 responses to BCG and, in some instances, reduce subsequent _M. tuberculosis_ burden [40–43]. Our current strategy to modulate OX40 signaling confers between a 1- and 4-log reduction in _M. tuberculosis_ burden of challenged mice compared with BCG alone. This dramatic enhancement in protection is superior to the vast majority of previous manipulations of BCG and is a promising strategy to improve protection against _M. tuberculosis_.

We must consider that OX40 is also expressed on other cell types and that OX40L:Ig may promote their function. NK cells produce excess IFN-γ when cocultured with OX40L-expressing dendritic cells. Their enhancement in OX40L: Ig-adjuvanted BCG-vaccinated mice that we report here is therefore likely to facilitate Th1 T-cell development [44]. Indeed, the superior protection to _M. tuberculosis_ observed in OX40L:Ig-treated mice was abolished with NK1.1+ cell depletion during BCG priming, suggesting a critical role for this cell type in OX40-dependent promotion of Th1 immunity. Despite the phagosomal location of both BCG and _M. tuberculosis_, CD8+ T-cell priming is also an important component of protective immunity and is thought to arise through cytosolic processing of _M. tuberculosis_ antigens [45]. Though originally thought not to impact on CD8+ T cells,OX40 is now recognized as promoting their activity either directly or indirectly via CD4+ T-cell help [12, 46]. The enhancement of activated CD8+ T cells in our study may therefore reflect direct activation by OX40L:Ig or additional help from the expanded CD4+ T-cell population.

One question that arises in proposing OX40 agonists as vaccine adjuvants is that if OX40:OX40L interactions are beneficial, why doesn’t endogenous OX40L on antigen-presenting cells suffice? We believe it is the length of time OX40L persists on APCs that determines the strength of the T-cell response. BCG causes downregulation of OX40L expression on thymic stromal lymphopoietin–stimulated dendritic cells in vitro [47]. Furthermore, mild infections (such as BCG) and attenuated, heat-inactivated or peptide-based vaccines may not provide a sufficiently robust innate stimulus to maintain OX40L expression. Optimal T-cell expansion and memory development requires 3 distinct signals: (1) antigen, (2) lipopolysaccharide or equivalent danger signal, and (3) ligation of OX40 [48]. The latter is clearly not maintained in response to BCG. This places an emphasis for successful vaccination on the cell presenting the vaccine.

In summary, we argue that BCG vaccination fails to maintain OX40L expression on APCs and thus does not maintain vaccine-activated T cells. Through ligation of OX40 with an OX40L:Ig fusion protein, we selectively boost recently activated mycobacterium-specific T-cell proliferation and Th1 cytokine production and development of an augmented memory response that is significantly superior to BCG in conferring protection to both intravenous and aerosol _M. tuberculosis_ infection. Intriguingly, NK 1.1+ cells appear to be critical in the OX40-mediated augmentation of immunity and protection, most likely through release of IFN-γ following engagement of OX40 on their surface and ensuing promotion of a Th1 immune response. Our results imply that sufficient innate stimulation is as important as T-cell priming in vaccine efficacy and that addition of molecules specific for late T-cell costimulators provides a targeted approach to overcome an attenuated response.

**Notes**

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