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Blockade of Allergic Airway Inflammation Following Systemic Treatment with a B7-Dendritic Cell (PD-L2) Cross-Linking Human Antibody

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We present a novel immunotherapeutic strategy using a human B7-DC cross-linking Ab that prevents lung inflammation, airway obstruction, and hyperreactivity to allergen in a mouse model of allergic inflammatory airway disease. Dendritic cells (DC) have the ability to skew the immune response toward a Th1 or Th2 polarity. The sHIgM12 Ab functions in vitro by cross-linking the costimulatory family molecule B7-DC (PD-L2) on DC up-regulating IL-12 production, homing to lymph nodes, and T cell-activating potential of these APCs. Using chicken OVA as a model Ag, the administration of sHIgM12 Ab to BALB/c mice blocked lung inflammation, airway pathology, and responsiveness to methacholine, even after animals were presensitized and a Th2-polarized immune response was established. This therapeutic strategy was ineffective in STAT4-deficient animals, indicating that IL-12 production is critical in this system. Moreover, the polarity of the immune response upon in vitro restimulation with Ag is changed in wild-type mice, with a resulting decrease in Th2 cytokines IL-4 and IL-5 and an increase in the immunoregulatory cytokine IL-10. These studies demonstrate that the immune response of hypersensitized responders can be modulated using B7-DC cross-linking Abs, preventing allergic airway disease upon re-exposure to allergens.

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2 S.R. and K.I. made equal contributions to this study.

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4 Abbreviations used in this paper: DC, dendritic cell; AHR, airway hyperreactivity; BAL, bronchoalveolar lavage; Penh, enhanced pause; pHIgM, polyclonal human IgM; sHIgM12, serum-derived human IgM Ab 12.
airway inflammation. Strikingly, this Ab treatment protected mice from allergic symptoms even when administered 14 days after hyper-sensitization. Moreover, the polarity of T cells isolated from the spleens of therapeutically treated mice was changed from strong Th2 to weak Th1. We conclude that B7-DC cross-linking Ab treatment protects mice in both a prophylactic and, more importantly, a therapeutic setting in a murine model of allergic airway inflammation.

Materials and Methods
Mice and reagents
Six- to 8-wk-old BALB/cJ and BALB/c-STAT4<sup>tm1Gru</sup> mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were maintained in the animal house facility, Mayo Clinic, as per the institutional guidelines for further studies. OVA protein was purchased from Sigma-Aldrich (St. Louis, MO). These studies were approved by the Mayo Clinic Institutional Animal Care and Use Committee. The IgM human Ab, sHlgM12, is derived from a patient with Waldenstrom’s macroglobulinemia, as described previously (11). Five hundred milligrams of Ab have been purified. The polyclonal human IgM (pHlgM) Ab used as a control in these studies has been described (18).

Immunoization and airway challenge
The sensitization and challenge procedure with OVA was modified from the method described by Zhang et al. (19). Briefly, mice were sensitized by an i.n. injection of 100 μg of OVA adsorbed to 1 mg of alum (Pierce, Rockford, IL). Experimental mice were intranasally challenged with 100 μg of OVA in PBS under tribromoethanol anesthesia.

Treatment with B7-DC cross-linking Ab
In the prophylactic regimen, mice were treated with sHlgM12 or the control pHlgM Ab i.v. at 10 μg per day on days −1, 0, and 1 before sensitization with OVA in alum. This schedule was designed to determine whether Ab treatment would prevent the establishment of a Th2-polarized response, typically elicited by the use of alum as an adjuvant. In the therapeutic regimen, the Ab treatments were conducted at the same dose and like condition in BALB/c mice. Mice were administered sHlgM12 Ab 1 day before, on the same day, and 1 day after immunization with OVA in alum (Fig. 1B). This regimen was designed to assess whether treatment with the sHlgM12 B7-DC cross-linking Ab results in skewing the immune response away from the pathogenic Th2 phenotype induced by immunization with the alum-adsorbed allergen.

Measurement of airway responsiveness to methacholine
Airway responsiveness was assessed by measuring methacholine-induced airflow obstruction in conscious mice in a whole body plethysmograph (Buxco Electronics, Troy, NY). Pulmonary airflow obstruction was measured by enhanced pause (Penh) with a transducer connected to pneumotaxi modules and analyzed by system software. To measure methacholine responsiveness, mice were exposed to 2 min of PBS followed by incremental dosages of aerosolized methacholine (Sigma-Aldrich). Penh was monitored for each dose.

Collection of bronchoalveolar lavage (BAL) fluid
Immediately after measuring airway hyperreactivity (AHR), animals were injected i.p. with a lethal dose (250 mg/kg) of pentobarbital (Abbott Laboratories, Abbott Park, IL). The trachea was cannulated, and the lungs were lavaged twice with 0.5 ml of HBSS. After centrifugation, the supernatant was collected and stored at −20°C. The cells were resuspended and counted using a hemocytometer. BAL cell differentials were determined with Wright-Giemsa stain. ≥200 cells were differentiated using conventional morphologic criteria. IL-5 in the BAL fluid supernatants was measured by ELISA, as directed by the manufacturer (R&D Systems, Minneapolis, MN).

Histology
After BAL fluid collection, the lung was fixed in 10% formalin and embedded in paraffin. Sections were obtained and stained with H&E, and in some cases with anti-CD3 Ab (using a peroxidase-labeled secondary developing reagent). The sections were evaluated by microscopy at ×100 and ×400 magnification.

In vitro cytokine production and proliferation
On day 27, splenocytes from the control Ab or the sHlgM12 Ab-treated mice were harvested and processed. Briefly, after making a single cell suspension, RBC were lysed by hypertonic shock using ammonium chloride/potassium bicarbonate/EDTA. Cells were counted and resuspended at 3 million cells/ml in RPMI 1640 (Cambrex, Walkersville, MD). OVA was made to a final concentration of 2 mg/ml and was titrated at half log dilutions. Supernatants were added at 3 × 10<sup>5</sup> cells in 100 μl. Supernatants were harvested after 48 h and stored for cytokine assay. Cells were pulsed with [3H]thymidine during the last 18 h of the 72-h assay. Cells were harvested and counted for incorporation of [3H]thymidine (Packard Instrument, Boston, MA).

stored supernatants were analyzed for IL-4, IL-5, IL-10, IFN-γ, and TNF-α by ELISA, per the manufacturer’s protocol (R&D Systems).

Statistical analysis
Data were analyzed using a two-way repeated measures ANOVA or Student’s t test for normally distributed data and the Whitney rank sum test for nonparametric data.

Results
Prophylactic treatment with sHlgM12 Ab prevents bronchial AHR in murine model of allergic airway inflammation
Treating bone marrow derived murine DC in vitro with sHlgM12 B7-DC cross-linking Ab resulted in secretion of IL-12 (12). IL-12 is a key cytokine that determines the polarity of the immune response; supporting the Th1 phenotype by promoting the secretion of IFN-γ, and contrasting the Th2 tendency of IL-4 and IL-5. Treating bone marrow derived murine DC with HlgM12 Ab resulted in skewing the immune response away from the pathogenic Th2 phenotype induced by immunization with the OVA-alum adjuvant combination in allergic disease such as asthma (13). To test whether cross-linking of B7-DC on murine DC with HlgM12 Ab results in skewing the immune response away from the pathogenic Th2 phenotype induced by immunization with the OVA-alum adjuvant combination, the effect of sHlgM12 Ab treatment during the initial immunization with OVA in the induced asthma-like condition in the BALB/c mice. Mice were administered sHlgM12 Ab 1 day before, the day of, and 1 day after immunization with OVA in alum (Fig. 1A). This regimen of Ab treatment results in significant reduction in airway responsiveness to methacholine challenge compared with the control Ab-treated mice. Mice treated with the sHlgM12 B7-DC cross-linking Ab, however, were significantly protected from airway responsiveness to methacholine challenge relative to animals treated with isotype control Abs (e.g.,...
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by Penh, as described in challenged with increasing dosages of methacholine. AHR was measured affected by a statistically

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flacterized to the lungs (Fig. 2C, p = 0.015). This failure to detect

significant eosinophilic infiltrates correlated with the reduced levels of IL-5 found following treatment with sHlgM12 Ab (Fig. 2D, p = 0.008). IL-5 is a cytokine that plays a pivotal role in migration of eosinophils (23). Most striking was the finding that sHlgM12 treatment totally abrogated lung inflammation, the thickening of bronchial epithelium, and the accompanying accumulation of mucus plugs that were readily evident in mice treated with isotype control Abs (Fig. 3, A and B).

FIGURE 2. Prophylactic effects of sHlgM12 B7-DC cross-linking Ab on OVA-induced AHR and inflammation. A, Responsiveness of mice to methacholine challenge. Mice that received either the isotype control polyclonal IgM Ab (○) or the B7-DC cross-linking Ab sHlgM12 (●) were challenged with increasing dosages of methacholine. AHR was measured by Penh, as described in Materials and Methods. Data are represented as means ± SEM (n = 10 per group). B, Cellular infiltration in lungs. Mice were sacrificed after measuring the AHR to methacholine. Cells present in BAL were counted. Data are represented as means ± SEM (n = 10/group). C, Eosinophilic infiltration in the lungs. The cells from the BAL fluid were stained with Wright-Giemsa for differential counts. Data are represented as the percentage of eosinophils with respect to the total number of cells counted. D, IL-5 in the BAL. The amount of IL-5 in the BAL was determined by ELISA. Data are represented as means ± SEM (n = 10/group). The OVA model of allergic asthma is characterized by pulmonary inflammation reflected by a statistically significant increase in the number of total cells in the BAL, an increase in the number of eosinophils in the BAL, and perivascular and peribronchial cellular infiltrates in lung tissue sections (22). The number of total cells in the BAL fluid was significantly reduced in sHlgM12-treated mice (Fig. 2B, p = 0.013). Moreover, sHlgM12 treatment also resulted in prevention of eosinophils migrating to the lungs (Fig. 2C, p = 0.015). This failure to detect significant eosinophilic infiltrates correlated with the reduced levels of IL-5 found following treatment with sHlgM12 Ab (Fig. 2D, p = 0.008). IL-5 is a cytokine that plays a pivotal role in migration of eosinophils (23). Most striking was the finding that sHlgM12 treatment totally abrogated lung inflammation, the thickening of bronchial epithelium, and the accompanying accumulation of mucus plugs that were readily evident in mice treated with isotype control Abs (Fig. 3, A and B).

Therapeutic treatment with sHlgM12 Ab prevents AHR

Next, we asked whether sHlgM12 Ab treatment could prevent mice from developing allergic airway inflammatory disease in a therapeutic model. In this model, the mice were treated with sHlgM12 Ab on days 13, 14, and 15 following initial sensitization with two priming doses of OVA in alum adjuvant (Fig. 1B). Inflammatory lung disease induced with this regimen was more severe than that induced with a single priming dose of OVA in alum adjuvant. This regimen of Ab treatment provided an opportunity to assess the potential of sHlgM12 Ab to modulate established immune reactivity, in a setting in which the immune response was already skewed toward a pathogenic Th2 polarity. Mice that received the sHlgM12 Ab showed a significant reduction in airway responsiveness to methacholine (Fig. 4A, p = 0.01) relative to animals that received isotype control Ab. The responses of sHlgM12-treated animals to methacholine were comparable to the responses of untreated, naive animals (Fig. 4A). Moreover, the number of cellular infiltrates in the BAL derived from sHlgM12-treated mice was markedly lower than the infiltrates found in BAL from either the control Ab or the PBS-treated mice (Fig. 4B, p = 0.008). The number of cells recovered in the BAL of sHlgM12-treated mice was comparable to the number recovered in normal

FIGURE 3. Lung pathology. All animals were sensitized with the experimental Ag chicken OVA using either the therapeutic or prophylactic protocols described in Fig. 1, with the exception of the mouse shown in C, which received no treatment. Lungs from all mice were fixed in formalin, and sections were stained with H&E. Data shown are representative of: A, BALB/c mice receiving isotype control IgM Ab during therapeutic regimen; B, BALB/c mice receiving sHlgM12 B7-DC cross-linking Ab, prophylactic regimen; C, naive BALB/c mice; D, BALB/c mice receiving PBS during therapeutic regimen; E, BALB/c mice receiving isotype control IgM Ab during therapeutic regimen; F, BALB/c mice receiving sHlgM12 B7-DC cross-linking Ab during therapeutic regimen; G, same as in E; H, same as in F; I, STAT4-deficient mice treated with isotype control IgM Ab; and J, STAT4-deficient mice treated with sHlgM12 B7-DC cross-linking Ab. Arrows point to areas of inflammation. Arrowhead points to bronchial epithelial thickening cause by airway inflammation. Three to ten animals were studied per group.
Treatment of mice with B7-DC cross-linking Ab. A, B7-DC cross-linking shHgM12 Ab treatment 13 days postadministration of Ag abrogates bronchial AHR. Mice that received either the isotype control polyclonal IgM Ab (O) or the shHgM12 B7-DC cross-linking Ab (●), and untreated, naive mice (▲) were subjected to airway hyperresponsiveness measurement in response to increasing dosage of methacholine, as in Fig. 1. Data are means ± SD (n = 5 per group). B, Absence of cellular infiltration in the BAL. After measuring the AHR, mice were sacrificed, BAL was extracted, and total cells in all the groups were counted. Data are means ± SEM (n = 5 in the Ab-treated groups, 6 in normal and PBS-treated groups). C, Prevention of eosinophil infiltration in the lungs. The cells from the BAL fluid were stained with Wright-Giemsa for differential counts. There were no detectable eosinophils either in the lungs of sHIgM12-treated mice or in the lungs of naive animals (Fig. 3C). In contrast, lungs from mice treated with PBS showed a significant infiltration of eosinophils (Fig. 3F). D, Reduced amount of IL-4 and IFN-γ in the lungs of sHIgM12-treated mice. The data are expressed as the percentage of eosinophils with respect to the total number of cells counted. E, Data are means ± SEM (n = 5 or 6 per group). F, Represents lungs from naive mice. G, Represents lungs from sHIgM12-treated mice. H, Absence of eosinophilic infiltration in lungs treated with sHIgM12 Ab. I, Systemic treatment with sHIgM12 Ab in allergic mice. Mice were sensitized with OVA in alum adjuvant. The splenocytes were stimulated in vitro for the nature of their recall response to OVA challenge to determine whether the Th2 response was altered toward a Th1 polarity. Mice were treated with sHIgM12 Ab or isotype control Ab on days 13, 14, and 15 post-sensitization with OVA in alum adjuvant. The splenocytes were

To further characterize the T cell response pattern, the supernatants from the homogenized lungs of the various groups of mice were analyzed for the prototypic Th1 cytokine IL-12 and the Th2 cytokine, IL-4. The mice that received shHgM12 showed significantly reduced amounts of both IL-4 (Fig. 4D, p = 0.008) and IFN-γ (Fig. 4E, p = 0.016) in comparison with the control Ab-treated mice. Although IL-5 levels were not measured in this experiment, the complete absence of eosinophilia suggests that IL-5 levels were most likely low in the shHgM12-treated animals. This cytokine pattern suggests that shHgM12 treatment does not lead to a proinflammatory cytokine environment in the lungs by switching the polarity of the T cell response from Th2 to Th1, but rather blocks the development of either kind of T cell response. Immunohistochemistry analysis of lung tissue using CD3-specific Ab as a probe supported this conclusion. Few T cells were present in the lungs of naive animals (Fig. 5A). In contrast, lungs from mice treated with PBS or polyclonal IgM control Ab contained extensive T cell infiltrates (Fig. 5, B and C). Remarkably, the lungs from shHgM12-treated mice showed no signs of infiltration (Fig. 5D), resembling the lungs of untreated mice (Fig. 5A). In addition, there was no lung pathology in animals that received the therapeutic treatment produced by B7-DC cross-linking sHgM12 Ab 14 days after presensitization; the lungs of naive animals (Fig. 3C) and shHgM12 Ab-treated mice (Fig. 3F) were indistinguishable. In contrast, animals that were treated with PBS (Fig. 3D) or isotype control Ab (Fig. 3E) exhibited severe distortion of their bronchial architecture and substantial inflammatory infiltration.

Our therapeutic strategy was predicated on our early observations that B7-DC cross-linking Ab induces IL-12 production by DC in vitro (12). To determine whether the IL-12 signaling pathways are important for the in vivo therapeutic effects of shHgM12 Ab treatment, we assessed the ability of Ab to modulate inflammatory airway disease in STAT4-deficient animals. STAT4 is a requisite intermediary that mediates IL-12 signaling (24). STAT4-deficient animals are known to develop highly polarized Th2 responses, as their ability to develop immune responses with Th1 character is severely compromised by the mutation (25, 26). The severity of induced airway inflammatory disease was substantially greater in STAT4-deficient animals than wild-type mice (Fig. 3G). Therapeutic treatment of the STAT4-deficient animals with B7-DC cross-linking Ab had no effect (Fig. 3J), while in the same experiment wild-type mice were completely protected from airflow inflammatory disease (Fig. 3H). This finding indicates that the ability to activate the STAT4 signaling pathway is critical for the treatment effect of shHgM12 Ab and provides mechanistic evidence that IL-12 may be important for altering the polarity of the response by presensitized animals.

TREATMENT WITH SHG12 ALTERS CYTOKINE PRODUCTION IN THE SPLEENS OF ALLERGEN-PRESENSITIZED MICE

In the absence of inflammation in the lungs of shHgM12-treated animals, we examined splenocytes from Ab-treated animals in vitro for the nature of their recall response to OVA challenge to determine whether the Th2 polarity characteristic of an allergic response was altered toward a Th1 polarity. Mice were treated with shHgM12 Ab or isotype control Ab on days 13, 14, and 15 post-sensitization with OVA in alum adjuvant. The splenocytes were
harvested at day 28 and were restimulated in vitro with OVA. The proliferative response of T cells in response to Ag was enhanced 10-fold in mice that had received sHlgM12 treatment in comparison with the control Ab treatment (Fig. 6A). This finding is consistent with our previous observation that treatment of DC with our B7-DC cross-linking Ab enhances their ability to stimulate T cells (11). Furthermore, it demonstrates the potential of this Ab to stimulate cellular responses against isolated proteins, an observation that may have important implications for the development of vaccines. The supernatants from the stimulated cultures were harvested and tested for the presence of cytokines. Although mice that received sHlgM12 produced significantly higher levels of IFN-γ than mice treated with isotype control Ab, neither treatment group produced substantial levels (Fig. 6B, p = 0.008). The same trend was observed for the amount of TNF-α produced, in which splenocytes from mice treated with sHlgM12 secrete small amounts of TNF-α, while no TNF-α was detected in control Ab-treated splenocytes (Fig. 6C, p = 0.029). In contrast to these Th1 cytokines, the prototypic Th2 cytokines IL-4 and IL-5 were substantially lower in cultures from mice treated with sHlgM12 Ab. Splenic cultures from mice treated with sHlgM12 contained very small quantities of IL-4 (Fig. 6D, p = 0.004) or IL-5 (Fig. 6E, p = 0.048). These data indicate that sHlgM12 skews the T cell response toward a Th1 polarity, but that even the Th1 response remains weak, despite a strong proliferative response to secondary Ag challenge. The presence of substantial levels of IL-10 in secondary cultures pretreated with the B7-DC cross-linking Ab sHlgM12 (Fig. 6F, p = 0.008) might explain the absence of inflammation in the lungs of mice challenged intranasally with experimental allergen. Despite the ability of T cells in these animals to secrete IFN-γ and TNF-α, T regulatory cells might dampen this tendency and prevent them from tracking to the lungs. Taken together, these data support the notion that sHlgM12 treatment protects presensitized individuals from allergic airway inflammatory disease by the ability of the Ab to prevent a Th2 type of environment and also due to its ability to promote the secretion of the anti-inflammatory cytokine, IL-10.

Discussion

The incidence and severity of allergic airway inflammation are increasing nationally and globally. The general improvement in hygiene during the last century may be responsible as the immune system seems to be unprepared by childhood infections. The hypothesis is that early childhood infection by viral infections leads to a predisposition later in life to develop allergic responses to inhaled environmental Antigens (28). We recently described a novel approach for the prevention of the allergic response to Ag using a human mAb that binds to mouse immune DC (11). This treatment differs from conventional Ab administration with CD40L, LPS, or CpG-oligodeoxynucleotides used to activate DCs in that maturation phenotypes induced by up-regulation of CD80, CD86, and MHC molecules are not induced. Animals treated with conventional DC activation experience splenomegaly, while mice treated with sHlgM12 Ab do not. Treatment of DCs with sHlgM12 Ab in vitro induces IL-12 and alters the migration pattern of DCs transplanted into mice (12). Remarkably, systemic treatment of mice with very small quantities (30 μg) of Ab can induce the same migration of DC to draining lymph nodes and potentiate Ag presentation of Ag-pulsed transplanted DC that reach the nodes (12).

The mechanism of DC activation by sHlgM12 Ab is not fully understood. We have shown that Ab binding to DC is dependent on expression of the costimulatory molecule B7-DC, and that B7-DC-binding ligands and B7-DC-specific IgG Abs can partially block the binding of the human mAb. Because monomers of the pentameric IgM Ab fail to activate, and in fact block, the activation by the native Ab, we have concluded that the cross-linking ability of the Ab is a critical feature of this reagent. We are now acquiring data (not described in this work) demonstrating conclusively that treatment of cultured DCs (both human and mouse) with sHlgM12 Ab activates specific signaling pathways and reproducibly up-regulates subsets of genes. These new findings tend to support the hypothesis that the systemic immunologic consequences of treatment with sHlgM12 are related to Ab-induced changes in resident DCs. Another, nonexclusive possibility is that Ab administered in vivo interferes with the interaction of B7-DC with a natural ligand. One such ligand is PD-1, a known negative regulator of T cell function (29). The possibility that additional undefined ligands exist is also plausible. Just how disruption of interactions between B7-DC and its ligands in vivo might influence the course of immunity is not known at present.

One possible explanation of the therapeutic effect of sHlgM12 is that B7-DC cross-linking by sHlgM12 Ab might lead to development of new sets of T cells that are skewed to secrete IFN-γ in response to IL-12 and IFN-γ secreted by the DC. This new lineage of T cells might then suppress the development of effector Th2
cells from the memory Th2 cell population, and, hence, IL-4 and IL-5 secretion in the vicinity of the lungs (30). The finding that STAT4-deficient mice are not responsive to sHlgM12 Ab treatment is consistent with this hypothesis. However, the possibility that STAT4 is an important signaling intermediary for a B7-DC ligand also remains open to question.

A recent report documents the ability of bone marrow-derived DC to secrete IFN-γ in response to IL-12 and skew the T cell response toward a Th1 polarity (31). However, we have only detected very small increases in IFN-γ production in these mice. Spleen cells from Ab-treated mice display a profoundly altered cytokine secretion pattern, suggesting that systemic Ab treatment changes in both species in vitro (S. Radhakrishnan and L. R. Pease, unpublished observations). This finding highlights the unique therapeutic value of our systemic treatment protocol using an IgM B7-DC cross-linking Ab.

An important feature of this model is its human origin for the sHlgM12 Ab. Importantly, the Ab binds human DCs as well as to mouse DCs, and induces comparable cellular and molecular changes in both species in vitro (S. Radhakrishnan and L. R. Pease, unpublished observations). Although it remains to be seen whether systemic treatment of human patients with this Ab can alter the course of asthmatic responses, this is an exciting possibility.

References
Letter of Retraction


In the course of investigating suspicious patterns of experimental results in the laboratory, a systematic and in-depth study of key findings in this article was carried out using blinded protocols. In these repeat studies, no evidence was found to support our original conclusions that B7-DC XAb modulates dendritic cell functions. We do not believe our failure to reproduce our earlier findings is the result of a technical problem. A member of the B7-DC XAb investigative team, Dr. Suresh Radhakrishnan, who was involved in or had access to all the work on this subject, was found in a formal investigation to have engaged in scientific misconduct in unpublished experiments involving the B7-DC XAb reagent. This finding of misconduct and our inability to reproduce key findings using blinded protocols has undermined our confidence in our published report. We seek, therefore, to retract this body of work.

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