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

Suresh Radhakrishnan, Koji Iijima, Takao Kobayashi, Moses Rodriguez, Hirohito Kita, and Larry R. Pease

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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3 Address correspondence and reprint requests to Dr. Larry R. Pease, Department of Immunology, Mayo Clinic College of Medicine, Mayo Clinic, 200 First Street SW, Rochester, MN 55905. E-mail address: pease.larry@mayo.edu
4 Abbreviations used in this paper: DC, dendritic cell; AHR, airway hyperreactivity; BAL, bronchoalveolar lavage; Penh, enhanced pause; pH1gM, polyclonal human IgM; s1gM12, serum-derived human IgM Ab 12.

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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 therapeu-tic setting in a murine model of allergic airway inflammation.

Materials and Methods

Mice and reagents
Six- to 8-wk-old BALB/c and BALB/c-STAT4tm1Gru 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, sHgM12, 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 (pHgM) Ab used as a control in these studies has been described (18).

Immunization 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 tribromethanol anesthesia.

Treatment with B7-DC cross-linking Ab

In the prophylactic regimen, mice were treated with sHgM12 on the control pHgM Ab i.v. at 10 μg per day on days −1, 0, and 1 relative to sensitization with OVA in alum. This schedule was designed to assess whether Ab treatment would prevent the establishment of the Th2-polarizing response, typically elicited by the use of alum as an adjuvant in the aper- tive regimen. The Ab treatments were conducted at the same dose and route on the day before the first intranasal challenge (Fig. 1A, day −1), and day after challenge with OVA in PBS (day 27). Results relative to first sensitization with OVA in alum. The pHgM Ab schedule was de-signed to assess whether treatment with B7-DC cross-linking Ab after im-munization with a Th2-polarizing regimen would modulate an estab-lished response polarity.

Measurement of airway responsiveness to methacholine

Airway responsiveness was assessed by measuring 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 pneumo-plier modules and analyzed by system software. To measure methacholine responsiveness, mice were exposed for 2 min to 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-Giemsas stained. ≅200 cells were differentiated using conven-tional morphologic criteria. IL-5 in the BAL fluid supernatants was mea-sured 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 em-bbeded in paraffin. Sections were obtained and stained with H&E, and in some cases with anti-CD3 Ab (using a peroxidase-labeled secondary de-veloping reagent). The sections were examined by microscopy at ×100 and ×400 magnification.

In vitro cytokine production and proliferation

On day 27, splenocytes from the control Ab or the sHgM12 Ab-treated mice were harvested and processed. Briefly, after making a single cell suspension, RBC were lysed by hypertonic shock using ammonium chlo-ride/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 di-lutions. Supernatants were added at 3 × 10⁶ 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 r test for normally distributed data and the Whitney rank sum test for nonparametric data.

Results

Prophylactic treatment with sHgM12 Ab prevents bronchial AHR in murine model of allergic airway inflammation

Treating bone marrow–derived murine DC in vitro with sHgM12 B7-DC cross-linking Ab resulted in secretion of IL-12 (12). IL-12 is a key cytokine in determining the polarity of the immune response by skewing T cell polarization toward a Th1 phenotype by promoting the secretion of IFN-γ and the induction of the Th2 toward Th1 cytokine trans-formation phenotype in allergic disease such as asthma (20). To test whether cross-linking of B7-DC on murine DC with pHgM12 Ab resulted in shifting the immune response away from the Th2-homing phenotype induced by immunization with the control alum adjuvant, we evaluated the effect of sHgM12 Ab treatment during the initial immunization with OVA in the induced asthma-like condition in the B6 mice. Mice were administered sHgM12 Ab 2 days before, the day of, and 1 day after immunization with OVA (Fig. 1A). This regimen of Ab treatment results in significant reduction in airway responsiveness to methacholine challenge as compared with the control Ab-treated mice. Mice treated with the sHgM12 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., pu-ri- nated human IgM (pHgM) Ab used as a control in these studies has been described (18).

FIGURE 1. Prophylactic and therapeutic Ab treatment schemes. A, Prophylactic treatment scheme. Mice received 10 μg of sHgM12 or isotype control polyclonal IgM Ab i.v. 1 day before, on the same day, and 1 day after the initial challenge with 100 μg of chicken OVA with the adjuvant alum. On day 14, animals received their first intranasal challenge with 100 μg of OVA. Beginning on day 23, animals received repeated intranasal challenges with OVA and were assayed for symptoms of allergic asthma on day 27. B, Therapeutic treatment scheme. Animals received two priming treatments with 100 μg of OVA, one on day 0, and the second on day 7. Treatments with sHgM12 Ab or polyclonal IgM isotype control Ab began on day 13, and were repeated on days 14 and 15. The intranasal challenges with 100 μg of OVA were on days 14, 23, 24, 25, and 26.
Fig. 2A, \( p = 0.041 \). 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 cell immune reactivity, in a setting in which the immune responses were 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
mice. Similar to our findings with the prophylactic treatment regimen, there was no detectable eosinophilic infiltration in the mice treated therapeutically with sHlgM12 Ab (Fig. 4F, p = 0.001).

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, IFN-γ, and the Th2 cytokine, IL-4. The mice that received sHlgM12 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 sHlgM12-treated animals. This cytokine pattern suggests that sHlgM12 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 sHlgM12-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 protocol of B7-DC cross-linking sHlgM12 Ab 14 days after presensitization; the lungs of naive animals (Fig. 3C) and sHlgM12 Ab-treated mice (Fig. 3F) were indistinguishable. In contrast, animals that were treated with PBS (Fig. 3D) or isotype control (Fig. 3E) exhibited severe distortion of their bronchial smooth muscle 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 sHlgM12 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 (Fig. 3I) relative to 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 airway inflammatory disease (Fig. 3H). This finding indicates that the ability to activate the STAT4 signaling pathway is critical for the treatment effect of sHlgM12 Ab and provides mechanistic evidence that IL-12 may be important for altering the polarity of the response by presensitized animals.

Treatment with sHlgM12 alters cytokine production in the spleens of allergen-presensitized mice

In the absence of inflammation in the lungs of sHlgM12-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 sHlgM12 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 sensitized 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 increasingly recognized. The general improvement in hygiene during the last century may be responsible as the immune system seems to be challenged by chance infections. The hypothesis is that during bacterial infection during childhood leads to a predisposition later in life to develop allergic responses to inhaled environmental Ads (28). We recently described a novel approach for the potentiation of the immune response to Ag using a human mAb that binds to mouse and human DC (11). This treatment differs from conventional methods of treatment 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 activating stimuli experience splenomegaly, while mice treated with sHlgM12 do not. Treatment of DCs with sHlgM12 Ab in vitro induced 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 alters the outcome of subsequent intranasal Ag challenge. Remarkably, no inflammation in the lungs occurs. The up-regulation of IL-10 production by splenocytes suggests the possibility that systemic treatment with sHlgM12 Ab may induce immunomodulatory regulatory T cells that suppress the effector responses in the lungs. A recent report demonstrates the ability of DCs to stimulate T regulatory cells in a costimulation-dependent fashion (32). The details of this kind of response still need to be established.

The possible involvement of PD-1, an inhibitory receptor for B7-DC (PD-L2) expressed by activated T cells, in immunomodulatory effects of sHlgM12 Ab treatment is not known. It remains possible that administration of the IgM Ab blocks interactions between DC and T cells, altering the course of T cell activation. However, it should be noted that very small quantities of Ab were administered during this treatment regimen and the affinity of the Ab for B7-DC is low. In previous experiments, DC treated in vitro with sHlgM12 Ab, and washed, retained the biological effects of treatment even when no Ab was detectable by flow cytometry on their cell surfaces (11). Therefore, we favor the hypothesis that the Ab acts by altering DC function, rather than by blocking interac-

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13. Lugo-Villarino, G., R. Maldonado-Lopez, R. Possemato, C. Penaranda, 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.
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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