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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-12 production. 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-12 production.

The hallmark feature of allergic asthma is abnormal expansion of Th2 cells in the lungs (2, 3). Dendritic cells (DC) act as major APCs to naive T cells in lymphoid organs (2, 3). Following presentation, the DC migrate to the draining lymph nodes, presenting Ag to CD4+ T cells, and activate T cell responses (4). Ag-specific T cells recognize the Ag presented by the DC via the major histocompatibility complex (MHC) class II molecules on the DC and require co-stimulation to trigger T cell activation (5, 6). The DC provide co-stimulation primarily via the B7 family receptors (B7-DC, B7-CD) and their ligands (B7-1, B7-2) (7, 8). The B7 family is involved in the regulation of T cell responses by controlling T cell priming and activation (7–10). These receptors are expressed on DC and other APCs and are required for T cell activation and the generation of an effective immune response.

We have previously demonstrated the ability of a human IgM mAb designated serum-derived human IgM Ab 12 (sHIgM12) to bind to both human and murine DC in an OVA (PD-L2)-dependent manner. This binding resulted in: 1) potentiation of the Ag-presenting ability of the DC as seen by the ability to activate OT-I TCR transgenic T cells; 2) increase in survival of the treated DC upon cytokine withdrawal; 3) secretion of IL-12; and 4) homing and/or survival of T cells, resulting in increased numbers reaching the draining lymph nodes. The loss in ability by a monomeric form of sHIgM12 mAb to potentiate the immune response, together with the finding that the monomer inhibits the activity of intact pentameric Ab, suggests that the targeted determinants on the surface of the DC are involved in cross-linking (11, 12).

B7-DC is one of the more recently identified costimulatory molecules belonging to the B7 family. B7-DC is expressed on DC and, upon activation, expressed on macrophages (13, 14). PD-1, the receptor for B7-DC (also known as PD-L2), is expressed on T cells upon activation, expressed on macrophages (13, 14). PD-1, the receptor for B7-DC (also known as PD-L2), is expressed on T cells upon activation and acts as a negative regulator as a consequence of an ITIM motif present in its cytoplasmic tail (15). However, this receptor-ligand interaction has been demonstrated to result in both inhibition of certain T cell responses (14) as well as activation of others (13). The dual nature of the responses elicited by B7-DC has been attributed to different kinds of stimulation used to modulate T cell function. However, it is also possible that B7-DC binds to additional receptors that mediate alternative functions. This hypothesis has been advanced recently in studies modeling the structure of the B7 family members and binding studies using T cells from PD-1-deficient mice (16, 17).

In this study, we examine the capability of our newly described B7-DC cross-linking Ab (11, 12) to modulate the immune response in vivo using an OVA model of murine allergic airway inflammation. Because cross-linking B7-DC on DC with sHIgM12 Ab resulted in secretion of IL-12 in vitro, a key cytokine that promotes Th1 responses, we reasoned that this Ab treatment might promote a change in the polarizing influence of DC in vivo, resulting in the protection of mice against allergic airway inflammation, and this protective effect was STAT4 dependent. Indeed, systemic administration of Ab before immunization completely protected mice from symptoms and lesions that mimic allergic
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/c and BALB/c-STAT4GluGlu 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 tribrethoanethol anesthesia.

Treatment with B7-DC cross-linking Ab

In the prophylactic regimen, mice were treated with sHgM12 or the control pHgM Ab i.v. at 10 μg per day on days −1, 0, and 1 before exposure 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 prophylactic regimen. The Ab treatments were conducted at the same dose and regimen, the Ab treatments were conducted at the same dose and regimen, and day after challenge with OVA in PBS (day 7) relative to first sensitization with OVA in alum. The BAL fluid was collected and stored at 0° C.

Histology

After BAL fluid collection, the lungs were 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 sHgM12 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 × 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).

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 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 the T cell population from a Th2 phenotype by promoting the secretion of IFN-γ, thereby making the T cell response from a Th2 toward Th1 phenotype. Previous reports in allergic disease such as asthma (20) suggest whether crosslinking of B7-DC on murine DC with sHgM12 Ab results in skewing the immune response away from the Th2-polarized response induced by immunization with the dominant allergen. We evaluated the effect of sHgM12 Ab treatment during the initial immunization with OVA in the induced asthma-like condition in the BALB/c mice. Mice were administered sHgM12 Ab 1 day before, the day of, and 1 day after immunization with 100 μg of OVA (Fig. 1A). This regimen of Ab treatment results in significant reduction in airway responsiveness to methacholine challenge in comparison 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.,

![FIGURE 1](https://www.jimmunol.org/))
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).
FIGURE 4. Therapeutic treatment of mice with B7-DC cross-linking Ab. A, B7-DC cross-linking sHlgM12 Ab treatment 13 days postadministration of Ag abrogates bronchial AHR. Mice that received either the isotype control polyclonal IgM Ab (Ο) or the sHlgM12 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 ± SEM (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 and in normal and PBS-treated groups). There were no detectable BAL in lung either the B7-DC cross-linking Ab-treated group or the normal mice. Data are represented as the percentage of eosinophils with respect to the total number of cells counted. D and E, Reduced airway eosinophilia and IFN-γ in the lungs of sHlgM12-treated mice. The lungs from naive or sHlgM12 Ab-treated mice were analyzed for T cell infiltration by histology using rabbit anti-CD3 Ab or sHlgM12 B7-DC cross-linking Ab. Arrows indicate sites of T cell infiltration marked by staining with anti-CD3 Abs. TheJournal of Immunology.

FIGURE 5. Systemic treatment with B7-DC cross-linking Ab blocks T cell inflammation in the lungs. Lungs from naive mice and mice treated therapeutically on days 14, 15 with PBS, control polyclonal IgM Ab or sHlgM12 Ab were analyzed for T cell infiltration. B7-DC cross-linking Ab 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. 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 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 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 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 increasing nationally (27). The general improvement in hygiene during the last several decades may be responsible as the immune system seems to be shut down by childhood infections. The hypothesis is that the failure of the infection during childhood leads to a predisposition later in life to develop allergic responses to inhaled environmental Ags (28). We recently described a novel approach for the potentiation of the immune response to Ag using a human mAb that elicits to mouse DC (11). This treatment differs from conventional in vivo 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 activation experience splenomegaly, while mice treated with sHlgM12 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 Th 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 sH IgM12 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 sH IgM12 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 observation that systemic regulatory T cells that suppress the effector responses in the lungs is consistent with our ability to block the release of IFN-γ by splenocytes from sHIgM12 Ab-treated mice (33). This finding highlights the unique therapeutic value of our systemic treatment protocol using an Ab that blocks B7-DC interactions.

An important feature of this model is the human origin of the sH IgM12 Ab. Importantly, the Ab binds human DCs as well as to mouse DCs, and induces comparable intracellular molecular changes in both species in vitro (S. Radhakrisnan 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 asthma 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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