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Repertoire-dependent Immunopathology

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Abstract

In humans, limited T-cell receptor repertoire and lymphopenia are associated with severe eosinophilic inflammatory disease. A model of lymphopenia and reduced T-cell repertoire was created; C57BL/6 Rag2^{-/-} mice received limited (30,000) or large (2 million) numbers of CD4 T-cells. Three to five months post-transfer, mice that had received 30,000 T-cells, but not those that received 2 million, developed fulminant macrophage pneumonia with eosinophilia, Ym1 deposition, methacholine-induced airway hyperresponsiveness, eosinophilic gastritis and esophagitis. These mice had strikingly elevated serum IgE (in CD3ε^{-/-} hosts) and donor-cells were enriched for IL-4, IL-5 and IL-13 producers. Th2 pathology and serum IgE were enhanced when transferred populations were depleted of CD25⁺ CD4 Tregs, but was more severe when the effector population was derived from limited as compared to the large effector population. Pretreatment of Rag2^{-/-} mice with 300,000 CD25⁺ CD4 Tregs prior to effector cell transfer prevented disease while pretreatment with 30,000 did not, despite the fact that there were equal numbers of Tregs in the hosts at the time of transfer of effector cells. Limited repertoire complexity of Tregs may lead to a failure to control immunopathologic responses and limited repertoire complexity of conventional cells may be responsible for the Th2 phenotype.

Keywords

IL-4; IL-13; IgE; alternatively activated macrophages; eosinophils

1. Introduction

In several human conditions associated with limited thymic output of T cells, striking immunopathology occurs usually associated with eosinophilic inflammation and with the production of Th2 cytokines. In such individuals, the diversity of T cell receptors expressed

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by peripheral T cells is often very limited. Examples of such immunopathology are observed in Omenn's Syndrome and in atypical complete DiGeorge Syndrome [1].

In the course of studying the factors responsible for determining whether rapid proliferation would occur when T cells were transferred from a donor to a recipient, we reached the conclusion that an important determinant of whether such proliferation would occur was the diversity of the T cell repertoires expressed by the recipients. In general, in recipients with equal numbers of CD44^{bright} CD4 T cells, rapid proliferation of transferred cells occurred in recipients that had TCR repertoires of limited diversity whereas those that had more complex TCR repertoires failed to support such rapid proliferation [2]. This led us to conclude that some type of specificity competition or repression occurs that limits the capacity of newly introduced cells to proliferate. In "empty" mice or in mice with a TCR population of limited complexity, there is a failure of the competition or repression and many newly introduced CD4 T cells divide rapidly.

Mice with repertoires of varying complexity but in which the numbers of CD44^{bright} CD4 T cells are similar were prepared by transferring varying numbers of CD4 T cells into Rag2^{-/-} recipients and waiting 4 to 8 weeks. At the end of that time, the mice had similar numbers of CD44^{bright} CD4 T cells and similar ratios of FoxP3⁺ and FoxP3⁻ CD4 T cells, but recipients of small numbers of CD4 T cells had limited repertoires when analyzed for CDR3 lengths in various Vb classes while recipients of large numbers of CD4 T cells had much more diverse Vb repertoires [2].

In order to determine whether a relationship between repertoire complexity and eosinophilic inflammatory immunopathology existed, we followed the recipient mice over an ~4 month period. We report here that these mice developed a fulminant macrophage/eosinophilic pneumonia and an eosinophilic gastritis.

2. Methods and methods

2.1 Mice

B10.A, Ly5.1 B10.A, C57BL/10, C57BL/10 AND TCR Tg, C57BL/10 Rag2^{-/-}, B10.A Rag2^{-/-}, B10.A CD3e^{-/-}, Ly5.1 C57BL/6, C57BL/6 Rag2^{-/-}, and C57BL/6 IL-4^{-/-} were obtained from the NIAID contract facility at Taconic Farms. C57BL/6 were obtained from Jackson Labs. Mice were maintained under pathogen-free conditions at the NIAID animal facility.

2.2 Adoptive transfer

CD4, CD25⁺ CD4, CD25⁻ CD4 or CD25⁻/CD44^{dull} CD4 lymph node cells were obtained by sorting on a FACSVantage SE or FACS Aria (BD). Purity was >99%. In some cases, cells were labeled with CFSE (Molecular Probes) at a final concentration of 1.25 μ M. Cells suspended in PBS were transferred via tail vein into recipient mice.

2.3 Flow cytometry

Anti-CD25-APC (PC61), CD4-FITC (3T4), CD44-PE, CD44 PE-cy5.5, CD45.1-PE, CD45.2-FITC, CD45.1 PE-cy5, IL-4-PE, IL-5-PE, IFN γ -APC were purchased from BD Pharmingen (San Diego, CA). Anti-FoxP3 was purchased from eBiosciences. Anti-IL-13 (clone 38213) was purchased from R&D and conjugated to APC at the NIAID core custom antibody facility. All flow cytometry was performed on BD FACSCaliber and analyzed using FloJo software.

2.4 Pathology/Immunohistochemistry

Immediately after euthanizing mice in a CO₂ chamber, organs were removed, fixed in neutral buffered formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin. Selected tissues were stained with Masson's trichrome (for collagen) or Luna stain (for eosinophils). Immunohistochemistry was performed on some tissues with antibodies to Ym-1 [3] by the ABC method (Vector Labs, Burlingame, CA). Anti-parietal cell antibodies were detected by immunofluorescence on cryostat sections of normal BALB/c stomach as described [4]. Briefly, sections were blocked with 2% fetal bovine serum in 5% dry milk in PBS and incubated with a 1/50 dilution of serum for 1 hour at room temperature. The presence of autoantibodies was visualized by adding FITC-goat F(ab')₂ anti-mouse Ig (Biosource). Slides were examined under a fluorescence microscope and given a score of 0–4 depending on the extent of parietal cell staining by an observer who did not have knowledge of the treatment the mice had received.

2.5 Methacholine hypersensitivity

Airway hyperresponsiveness of control Rag2^{-/-} mice and recipients of cell transfers was measured by challenging the mice with increasing doses of nebulized methylcholine (0, 6 and 12 mg/ml) in PBS and measuring "enhanced pause" using whole body plethysmography (Buxco Electronics) following the manufacturer's instructions. Doses above 12 mg/ml resulted in significant morbidity in compromised mice consistent with asphyxia.

2.6 IgE ELISA

Immulon 4 96 wells microtiter plate were coated with 100 µl/well of anti-mouse IgE (Pharmingen) at 2 mg/ml in PBS overnight at 4 degrees. Plates were washed with wash buffer (PBS +0.05% Tween20) and blocked for one hour at room temperature with diluent/blocking buffer (PBS + 0.5% bovine serum albumin (BSA) + 0.05% tween 20). Samples (1/50 or 1/500 dilutions of mouse serum or mouse IgE standard (Pharmingen) were added and incubated for two hours at room temperature. Plates were washed, and biotin-anti mouse IgE (Pharmingen) was added in diluent/blocking buffer for two hours at room temperature. Plates were washed again and streptavidin-HRP (Pharmingen) (1/4000 dilution in diluents/blocking buffer) was added for one hour. Plates were washed again, TMB solution (Sigma) was added for 2–3 minutes, and Stop Solution (2N H₂SO₄) was then added. Plates were read at 450 nm and the optical densities of the samples were then plotted on the linear part of the standard curve and multiplied by the dilution factor in order to calculate IgE concentration in serum samples.

3. Results

CD4 T cells from C57BL/6 mice were transferred into C57BL/6 Rag2^{-/-} recipients. Animals received either 30,000 or 2 million cells and were observed over a period of up to six months. In recipients of 30,000 cells, a fulminant macrophage pneumonia developed, characterized by infiltrations of eosinophils and alternatively-activated macrophages that could be seen to contain microcrystals consisting of YM1 (chitinase) (Figure 1). Microcrystals were also found in extracellular spaces. This occurred in 13 of 13 mice examined. By contrast, none of 7 recipients of 2 million CD4 T cells developed this type of pneumonia (Figure 2). The lung lesions of affected mice were also characterized by mucus metaplasia of the airway epithelium.

The affected animals also showed methacholine airway hypersensitivity whereas the control animals did not.

In addition to lung lesions, the recipients of 30,000 CD4 T cells developed eosinophilic gastritis with loss of parietal cells and eosinophilic inflammatory responses in other organs. Mice that received 2 million cells did not develop these inflammatory responses.

In the lymph nodes of recipients of 30,000 cells, there was a striking increase in the proportion of cells that were prepared to produce IL-5, IL-13 and IL-4 upon stimulation when compared to mice that had received 2 million cells. No differences in the capacity of the cells to produce IFN γ was noted.

When CD3e^{-/-} mice were chosen as recipients, mice that received 30,000 cells showed striking elevation of serum IgE levels by 8 to 12 weeks. Mean values of ~100 mg/ml were observed whereas recipients of 2 million cells had values barely above background levels.

These results indicate that T cell deficient mice developed a severe TH2-type inflammatory disorder when they receive a limited number of purified CD4 T cells. We further observed that increases in serum IgE were even greater in mice that had received CD4 T cells from which CD44^{bright} and CD25^{pos} cells had been removed, suggesting that naïve conventional CD4 T cells were responsible for the disorder and raising the possibility that the response was restrained by the presence of regulatory T cells.

To test the possible role that regulatory T cells played in restraining this immunopathology, we transferred 30,000 or 300,000 purified CD25⁺ CD4 T cells into Rag2^{-/-} recipients. Consistent with reports by Freitas and colleagues [5], we observed that the frequency of FoxP3⁺ cells in the recipients 6 weeks after transfer was similar in mice that had received 30,000 or 300,000 cells. Thus, it appears that regulatory CD4 T cells reconstitute recipients to some fixed number and, thus, recipients of smaller numbers of cells should have a simpler repertoire than recipients of large numbers of regulatory CD4 T cells.

We then asked whether mice that had received small number of purified regulatory T cells were similar to or different from mice that had received large numbers of regulatory T cells in their capacity to restrain expansion of newly transferred CD4⁺ T cells. Indeed, there was a striking diminution in the degree of expansion of the transferred cells in the animals that had received 300,000 CD25⁺ cells compared to those that received 30,000 CD25⁺ T cells. More strikingly, the initial transfer of 300,000 CD25⁺ CD4⁺ T cells protected recipients against the “Th2-type” immunopathology observed in mice that received no pre-transfer. However, pre-transfer of 30,000 CD25⁺ CD4 T cells did not protect mice against the immunopathology mediated by 100,000 conventional CD4 T cells. Thus, it appears that a repertoire complex population of regulatory CD4 T cells can protect mice against the immunopathology that develops when limited numbers of conventional CD4 T cells are introduced into an “empty” recipient; however, a repertoire simple population of regulatory CD4 offered little protection.

4. Discussion

Our prior studies and those of others have led that introduction of CD4 T cells into lymphopenic recipients leads to proliferative responses. In general, one observes that some of the introduced cells undergo one or two divisions in a seven day period. This proliferation is inhibited by antibodies to the IL-7 receptor alpha chain, implying that it is IL-7 dependent. Other cells show a much more vigorous proliferative response. In a 7 day period, many of these cells divide 7 times or more and acquire a CD44^{bright} phenotype, implying they have become memory or effector/memory T cells. This rapid proliferative response is not inhibited by anti IL-7Ra but is blocked by anti-class II MHC antibodies, implying that it is a TCR-dependent IL-7 independent response [6].

In general, monoclonal CD4 T cells of one specificity do not prevent the proliferation observed when polyclonal CD4 T cells are introduced into Rag2^{-/-} recipients and polyclonal CD4 T cells show rapid proliferation when introduced into TCR transgenic Rag2^{-/-} recipients. Furthermore, when recipients are prepared that have a relatively simple TCR repertoire, they allow newly introduced cells to proliferate whereas mice that had more complex repertoires

(although they have similar numbers of CD44^{bright} CD4 T cells), restrain the proliferation of newly introduced CD4 T cells [2].

These results imply that the proliferation of newly introduced cells is either inhibited by some type of specificity related competition or by specificity-related suppressor activity.

By observing recipients of limited numbers of CD4 T cells for a longer period, we noted the development of a severe Th2-type immunopathology in mice that received small (30,000) but not large (2 million) CD4 T cells. This led us to conclude that the mechanisms that limited proliferation of newly introduced CD4 T cells might also be important in preventing the development of Th2 type immunopathology with its attendant eosinophilic inflammation. Indeed, we showed here that a complex population of regulatory CD4 T cells strikingly inhibited the development of eosinophilic inflammation but a repertoire simple population of regulatory CD4 T cells did not.

There are several interesting aspects of the immunopathology observed. Firstly, since it occurs regularly (in 13 of 13 mice) despite the very limited repertoires that these mice possess, it seems unlikely that it represents a response against a single autoimmune target antigen. For example, in the autoimmune gastritis seen in three-day thymectomized mice, which is believed to be due to a relative paucity of regulatory T cells, one of the key autoantigens in the NA/K ATPase expressed in parietal cells [7]. While one cannot be certain, it would seem that the limited repertoire in mice that had received 30,000 cells would be too small for CD4 T cells specific for such an antigen to routinely emerge. Indeed, one can estimate the size of the repertoire in recipients of 30,000 cells. In our experience, the efficiency with which injected cells are actually capable of taking up residence in the spleen or lymph nodes of the recipient is under 10%. Further, not all transferred cells undergo strikingly proliferation. While we do not have a firm frequency for cells capable of such proliferation, we suspect it is well under 10%. Using these estimates, the number of cells that actually undergo vigorous proliferation in recipients of 30,000 cells may be no more than 300. With such a limited repertoire, it would seem very unlikely that auto-antigen-specific cells would regularly be present.

We think it more likely that cells undergoing vigorous proliferation will also make effector cytokines in the tissue site in which they are dividing. It is clear that the simple presence of Th2 cytokines such as IL-4, IL-13 and IL-5 in the lung can acutely lead to mucus metaplasia, airway hypersensitivity and eosinophilia [8]. What the consequences of the chronic presence of these cytokines would be is not known but the infiltration/induction of alternatively activated macrophages is certainly a possibility since IL-4 and IL-13 are known to cause the differentiation of macrophages to this phenotype. Certainly, these cytokines and IL-5 would contribute to eosinophilic infiltration.

Why does a repertoire complex population of regulatory cells control the development of immunopathology but a repertoire simple population fail to do so? We would argue that the capacity of regulatory T cells to efficiently inhibit the response of conventional cells would depend on the two cell types being activated in the same place at the same time. Indeed, it can be anticipated that regulatory T cells would most efficiently control the behavior of conventional CD4 T cells if both were activated by the same dendritic cell at the same time. This therefore requires that both the conventional cell and the regulatory cell recognize peptide/MHCII complexes presented by the same tissue resident or lymph node resident DC and this would be more likely to occur when the two populations are both complex and more importantly when the regulatory population has a complex repertoire.

Why is the inflammatory response observed a Th2 type response? It is possible that in the absence of regulatory CD4 T cells, Th2 response predominate. However, what is observed in mice in which the FoxP3 gene is disrupted is an intense multiorgan inflammatory response

associated with allergic airway inflammation, a striking hyperimmunoglobulinemia E, eosinophilia, and dysregulated Th1 and Th2 cytokine production in the absence of overt Th2 skewing [9]. In humans with the IPEX syndrome, lymphoproliferation, autoimmunity, and intense allergic inflammation occur, but the response is by no means limited to a Th2 response [10]. This implies that while the absence of regulatory T cells allows for a Th2-type inflammatory response, it is not uniquely a Th2 response. In our model, the immunopathology observed thus far has largely been eosinophilic inflammation with elevation of IgE. However, among mice that receive 30,000 CD4 T cells, IFN γ -producing CD4 T cells are observed in a frequency similar to that in recipients of 2 million cells. Direct tests for the relative sensitivity of developing Th1 and Th2 cells to regulatory effects need to be carried out.

Limited repertoires by themselves could favor Th2 responses. Under various conditions, stimulation of naïve transgenic CD4 T cells with peptide/MHC complexes of low affinity for the TCRs or with low peptide concentrations leads to striking TH2 responses which are lost as affinity or concentration are increased [11]. We have reported that vigorous T cells stimulation leads to erk activation which represses GATA3 and IL-4 production and thus blocks Th2 responses [12]. In situations in which repertoires are of limited complexity, it might be expected that a high affinity peptide/MHC- TCR interaction would be unlikely and that the dominant response would therefore be skewed to the Th2 phenotype. Alternatively, it has been suggested that the high degree of proliferation seen in animals that have limited repertoires might favor a Th2 response because of less dramatic apoptosis in these cells and because of the proliferative advantage Th2 cells often have over Th1 cells [13]. In order to examine this possibility, the analysis of additional models of limited repertoire not dependent upon transfer of limiting numbers of founders would be most helpful. The discussion herein is unique amongst the papers presented in this meeting and we note for completeness several other aspects of loss of tolerance that are discussed in this special issue, including the role of natural autoantibodies, Tregs, apoptosis, and autophagy [14–27]

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Abbreviations

TCR

T-cell receptor

CDR3

third complementarity determining region

Th

	T-helper
IL	interleukin
MHC	Major histocompatibility complex
TCR	T-cell Receptor

Figure 1.

Luna stain for eosinophils of lung sections from Rag2^{-/-} mice reconstituted with 30,000 CD4⁺ four months earlier. Large macrophages containing eosinophilic crystals are visible, as are smaller eosinophils containing multilobed nuclei.

Figure 2.
Luna stain for eosinophils of lung sections from Rag2^{-/-} mice reconstituted with 1 million CD4⁺ four months earlier.